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Zinc to the sink: Genetics of increased Zn in maize kernels

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Zinc to the sink: Genetics of increased Zn in maize kernels

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The student author and the program of study committee are solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Zinc (Zn) deficiency is a global health problem particularly in low- and middle-income countries where diets are cereal-based and typically lower in Zn. Biofortification, the genetic enhancement of staple foods through plant breeding is considered cost-effective and sustainable. Maize is one of the major crops grown and consumed in the regions where Zn deficiency is prevalent. But Zn concentration in maize kernels is insufficient to meet the requirements of humans. Therefore, breeding varieties with increased Zn concentration is an important goal for maize breeders.

To breed Zn-biofortified varieties, it is imperative to identify germplasm with high-Zn concentration, assess their potential for grain yield and other important traits, and develop knowledge-based strategies for artificial selection. Two separate studies were conducted to assess the potential of improving maize adapted to the tropics for kernel Zn concentration.

Study 1: Twenty elite inbred lines (10 quality protein maize (QPM) and 10 non-QPM) were systematically mated using a modified mating design. The generated hybrids were evaluated for kernel Zn, grain yield and flowering time in field experiments across four environments. Statistical analyses with respect to the mating design were implemented and hybrids with high-Zn and grain yield were identified. General combining ability (GCA) effects for Zn concentration were more preponderant than specific combining ability (SCA) effects, suggesting the importance of additive gene action for kernel Zn inheritance.

Study 2: An association mapping panel and two bi-parental populations, evaluated for Zn concentration in three environments were used to assess the feasibility of genomic prediction for kernel Zn. Two distinct cross-validation schemes (CV1 and CV2) simulating two genomic prediction breeding scenarios were used to estimate the prediction ability (r_{MP}) for Zn. Prediction

accuracy values ranging from 0.51 to 0.71 were observed indicating the potential of genomic prediction for biofortification breeding to enhance Zn concentration in tropical maize germplasm.

CHAPTER 1. GENERAL INTRODUCTION

Zinc (Zn) is a trace element found in variable concentrations in soils, plants, and animals. If the amount of Zn available is not adequate, plants and/or animals will suffer from physiological stress related to dysfunction of several enzymatic systems and other metabolic functions in which Zn is involved [1]. Among the trace elements, Zn is needed by the largest number of proteins [2,3]. In eukaryotic cells, one-tenth of the proteome (complete set of proteins expressed by an organism) are from Zn-containing proteins and 36% are involved in some aspects of gene expression [4].

Zn is an essential micronutrient needed by the human body in small quantities, generally less than 100 mg per day [5]. Intake recommendations for Zn are developed by the Food and Nutrition Board (FNB) at the Institute of Medicine of the National Academy of Sciences [6]. Zn is naturally present in some foods, added to others and available as a dietary supplement. A wide variety of foods can supply Zn (Table 1.1**)**, and foods providing ≥ 20% of the daily value (DV) are high sources.

Table 1.1 Selected food sources of zinc (USDA,2011)

Food	Milligram (mg) per serving	Percent DV*
Oysters, cooked, breaded and fried, 3 ounces	74	493
Beef chuck roast, braised, 3 ounces	7	47
Crab, Alaska king, cooked, 3 ounces	6.5	43
Beef patty, broiled, 3 ounces	5.3	35
Breakfast cereal, fortified with 25% of the DV for zinc, ¾ cup serving	3.8	25
Lobster, cooked, 3 ounces	3.4	23
Pork chop, loin, cooked, 3 ounces	2.9	19
Baked beans, canned, plain or vegetarian, ½ cup	2.9	19

Table 1.1 continued.

Food	Milligram (mg) per serving	Percent DV*
Baked beans, canned, plain or vegetarian, ½ cup	2.9	19
Chicken, dark meat, cooked, 3 ounces	2.4	16
Yogurt, fruit, low-fat, 8 ounces	1.7	11
Cashews, dry roasted, 1 ounce	1.6	11
Chickpeas, cooked, ½ cup	1.3	9
Cheese, Swiss, 1 ounce	1.2	8
Oatmeal, instant, plain, prepared with water, 1 packet	1.1	7
Milk, low-fat or nonfat, 1 cup	1	7
Almonds, dry roasted, 1 ounce	0.9	6
Kidney beans, cooked, ½ cup	0.9	6
Chicken breast, roasted, skin removed, ½ breast	0.9	6
Cheese, cheddar or mozzarella, 1 ounce	0.9	6
Peas, green, frozen, cooked, ½ cup	0.5	3

DV = Daily Value. This value helps to compare the nutrient contents of products within the context of a total diet. The DV for zinc is 15 mg for adults and children four years and older.

Maize (*Zea mays* L.) is an important cereal crop, contributing nearly 35% of total cereal production in the world [7]. Globally, maize occupies ~197.2 million hectares with a production of 1134.7 million tones [7] . The utility of maize as food is expected to increase mainly in developing countries because of the growing populations and increasing importance of especially white maize in diets [8]. Maize contains about 72% starch, 10% protein and 4% fat accounting for roughly one quarter of the total caloric intake relative to rice and wheat [9,10].

Maize kernels may supply many of the macro- and micronutrients necessary for human metabolic needs. However, the amounts of some essential nutrients, including Zn are inadequate for consumers who rely on maize as a staple food [10]. In a maize kernel, the mean Zn concentration is ~ 2 mg /100 g of grain [11]. A range of between 1.29 and 5.76 mg /100 g of kernels has been reported [12]. Processing and consumption of maize varies greatly with maize flour/meal being the most popular product.

Many people in developing countries do not eat a balanced diet because they rely on staple foods such as maize which are low in Zn leading to malnutrition [13]. To mitigate the effects of malnutrition, plant breeders are developing maize varieties with enhanced kernel Zn through a process known as 'biofortification' [14]. When consumed regularly, Zn-biofortified maize will generate measurable improvements in human health and nutrition [15].

The process of breeding Zn-enriched varieties entails screening germplasm for available genetic diversity, developing and testing Zn-enriched inbreds, conducting genetic studies, and developing knowledge-based strategies for artificial selection such as genomic prediction to lower costs, enhance efficiency and hasten the process of breeding [15]. The biofortified varieties should then be tested in multiple locations and years to assess genotype by environment (G x E) interaction.

Literature Review

Importance of Zn in human nutrition and metabolism

Zinc is an essential element for human health and a key component of many enzymes in the body [16]. Zn serves as a cofactor for more than 300 enzymes involved in the synthesis and degradation of carbohydrates, lipids, proteins, and nucleic acids as well as in the metabolism of other micronutrients [17]. Zn has also been shown to play vital roles in DNA transcription and translation perhaps accounting for the importance of Zn to all forms of life [18]. Zn is essential for tissue growth, healing of wounds, proper function of the immune system, bone mineralization, prostaglandin production, proper thyroid function, fetal growth, sperm production and maintenance of normal serum testosterone [19].

Intake recommendations for Zn varies by age and gender (Table 2.1) [20,21]. For instance, the human body requires more Zn during periods of rapid growth like infancy,

pregnancy and adolescence [19,22]. In adolescents, the physiological requirements for Zn increases at puberty, generally occurring between 10-15 years in girls and between 12-15 years in boys [23].The role of maternal Zn status on pregnancy outcome is still unclear. Positive and negative associations between plasma Zn concentration and fetal growth or labor and delivery complications have been reported [24].

Additionally, requirements for dietary Zn are determined partly by the physiological processes governing tissue demands for Zn, the rate of loss from the body, and by the intrinsic characteristics of the diet [25]. Requirements are markedly increased when infants and young children are recovering from malnutrition or infection [26]. For most age groups, a factorial method is used to estimate the average physiological requirement, defined as the amount of Zn that must be absorbed to offset the amount of Zn lost through both intestinal and non-intestinal pathways. In growing children and pregnant women, the amount of Zn retained in newly accrued tissues is added to the requirements, and for lactating women, the Zn secreted in breast milk is added [27].

Zn is present in all organs, tissues and fluids of a human body. However, the body has no specialized storage system for Zn and a daily intake of Zn is required to maintain a steady state [28]. The average daily level of intake sufficient to meet the nutrient requirements of a healthy individual (recommended dietary allowance [RDA]) ranges from 2 mg (infants 0-6 months) -11 mg (adults 19 + years). The total body Zn of an adult human is approximately 1500-2000 mg (~0.003% of total body weight) [19]. For infants, recommended intakes of Zn are based on an Adequate Intake (AI). AI is established when there is no sufficient evidence to develop RDA and is set at a level assumed to ensure nutritional adequacy [20].

Table 1.2 Estimated physiological requirements for absorbed Zn by age group and gender

	WHO			FNB		IZiNCG	
Variable age	Reference weight(kg)	Requirement (mg/day)	Variable age	Reference weight(kg)	Requirement (mg/day)	Reference weight(kg)	Requirement (mg/day)
6-12 months	9	0.84	6-12 months	9	0.84	9	0.84
1-3 years	12	0.83	1-3 years	13	0.74	12	0.53
3-6 years	1	0.97	4-8 years	22	1.2	21	0.83
6-10 years	25	1.12	8-13 years	40	2.12	38	1.53
10-12 years	35	1.40	14-18 years (male)	64	3.37	64	2.52
12-15 years	48	1.82	14-18 years (male)	57	3.02	56	1.98
15-18 years (male)	64	1.97	Pregnancy		4.1-5.0		2.68
15-18 years (female)	55	1.54	Lactation		3.8-4.5		2.98
Pregnancy		2.27					
Lactation		2.89					

WHO=world health organization, FNB=food and nutrition board, IZiNCG= International Zinc Nutrition Consultative Group

Inadequate intake of dietary Zn is the primary cause of Zn deficiency particularly in populations depending on diets low in absorbable Zn. Zn deficiency becomes a problem because these populations consume mainly cereals with no or limited access to alternative food sources like meat that are rich in bioavailable Zn [29]. Other causes for Zn deficiency include malabsorption, increased losses and impaired utilization [19]. In the case of an infection, utilization of Zn is impaired and its availability to tissues reduced [23]. Zn deficiency is characterized by growth retardation, loss of appetite, delayed sexual maturation and impaired immune function [30–32].

Approximately, 20% of the world's population is at risk of inadequate Zn intake and supply [34]. The risk ranges from, 9% in the regions of Eastern Mediterranean, North Africa, USA, and Canada to 33% in Southeast Asia, Latin America and sub-Saharan Africa [34,35]. The amount of absorbable Zn in food supplies is greater in the more industrialized countries of Western Europe, USA and Canada than in the lower-income countries of Latin America, Southeast Asia and Sub-Saharan Africa [34]. Zn deficiency has, therefore, been identified as a high priority and of global importance. Eliminating the deficiency will result in immediate high impacts and returns for humanity within populations where Zn deficiency is prevalent [35–37].

In the body, Zn absorption is influenced by (i) the amount of Zn present in the intestinal lumen (ii) the presence of dietary promoters (e.g. animal proteins) or inhibitors (e.g. phytate and other minerals) and (iii) the physiological state of an individual (e.g. pregnancy, lactation and early infancy) [38]. Zn is released from food as free ions which may then bind to endogenously secreted ligands before their transport into the small intestines [39]. Specific transport proteins (*ZnT*) may facilitate the passage of Zn across the cell membrane into the portal circulation [40].

The blood vessels carry absorbed Zn directly to the liver where it is released into a systemic circulation for delivery to other tissues.

The ability to assess the nutritional status of a population is critical in efforts to develop an intervention program for Zn deficiency. Assessment methods can be grouped into: (i) those that provide suggestive evidence for the risk of Zn deficiency in populations and (ii) those that are applied to specifically estimate the risk of Zn deficiency in a population. Suggestive evidence includes stunting (low height-for-age) among pre-school children and is a common clinical manifestation of Zn deficiency. Although stunting can be caused by other health or environmental factors, an elevated prevalence of the condition may be used as an evidence of Zn deficiency in a population [23]. The concentration of Zn in blood plasma or serum can be used to assess Zn deficiency. Zn deficiency will be considered a public health concern when low serum Zn concentration is prevalent in >20% of the population [23].

A wide range of health benefits can be realized by increasing the intake of Zn where diets are inadequate in this micronutrient [41,42]. Major intervention strategies are supplementation, dietary diversification, fortification, and biofortification. Dietary diversification can be a sustainable long-term approach to improve intake of several nutrients simultaneously [43]. Supplementation programs are useful for targeting populations at high-risk of Zn deficiency. Zn supplements could be included in programs already delivering daily or weekly nutrient supplements for the prevention of other deficiencies such as iron [44]. In populations where Zn deficiency is common, fortification of centrally processed foods can be appropriate and other micronutrients can also be added. However, for such multiple interventions synergistic and antagonistic interactions between micronutrients have to be taken into account during the development of appropriate formulations [23].

These intervention strategies have worked well in developed countries. However, the interventions may not be appropriate for a majority of the populations where Zn deficiency is prevalent because they produce their own food on their farms. Hence, biofortification is considered a viable solution because higher Zn concentration and availability will be bred into the varieties. Biofortification seeks to take advantage of the consistent and daily consumption of staple foods by all family members particularly for low-income households [45]. Maize cultivars with increased Zn concentration can thus, provide at least part of the nutritional requirements and promote self-reliance for those populations [46].

Importance of Zn in plant physiology and metabolism

Even though needed in lesser amounts, Zn has a significant impact on how a maize plant grows and ultimately how much yield is produced. Zn plays a very critical role in (i) the synthesis (production) of growth hormones and proteins, (ii) the production of chlorophyll and carbohydrate metabolism, (iii) transportation of calcium throughout the maize plant (iv) cell elongation and (v) increase in leaf and node size along with grain formation [47]. Zn is, therefore, very important as a structural constituent and a regulatory cofactor of a wide range of different enzymes and proteins in many important biochemical pathways of a maize plant. These include carbohydrate metabolism, both in photosynthesis and in the conversion of sugars to starch, protein metabolism, auxin (growth regulator) metabolism, pollen formation, maintenance of membrane integrity and the resistance to infection by certain pathogens [48,49].

Zn is absorbed by roots primarily as Zn^{2+} from the soil solution and its uptake is mediated by Zn-regulated transporters [48,50]. From the roots, Zn is transferred to the vascular bundles for transport to the above-ground tissues [51]. Studies on *Arabidopsis* have shown that the heavy metal, ATPase (*HMA*) genes expressed in the vascular tissue are the most likely candidates for

transporting Zn [52–54]. During transportation, Zn is chelated by nicotianamine (NA), a metal chelating molecule present in both the xylem and phloem [55]. Yellow stripe-like transporters (YSLs) are also often proposed to be involved in the transport of chelated Zn from the xylem into the phloem so that Zn can be distributed to the young growing tissues of the plant [56].

Patterns of mineral assimilation are nutrient specific and vary in the timing, rate, duration of uptake as well as the tissues to which nutrients are partitioned. Furthermore, nutrients exhibit varying degrees of mobility within the plant once assimilated into a tissue [57]. Studies evaluating the time course of Zn and its partitioning considered four parts of the maize plant: leaves, stems, cobs and kernels. In leaves, the highest Zn concentrations were found at the vegetative stage of maize growth, i.e. stage of 7^{th} leaf up to 9^{th} after which the concentration of Zn in leaves showed a declining trend. From the 9^{th} leaf stage to tasselling stage, a large decrease in Zn content in leaf and a simultaneous increase of Zn in the stem occurs. This is an indicator of Zn remobilization from the leaves. Translocation of Zn to the grain starts at the R2 growth stage. This stage occurs 10 – 14 days after silks emerge [57]. Extensive remobilization of Zn from vegetative organs occurs at the beginning of the R4, or 'dough' stage of kernel development [58]. By R6 (physiological maturity stage), nearly 60% of stalk Zn will have been remobilized to the grain [59].

The kernel obtains Zn by two mechanisms: (i) transfer of Zn from the phloem to the grain through the xylem, which also involves senescence based remobilization and (ii) direct transfer of Zn from the phloem to the developing seed [60,61]. However, the level of remobilization and efficiency of direct transfer may vary with genotypes. NAC (NAM, ATAF, and CUC) transcription factor (NAM-B1) which is associated with plant stress response has a major role in regulating key genes for the early onset of senescence, which results in higher Zn concentrations

in grains [62]. Therefore, a significant part of the total mineral content including Zn in the kernel comes from remobilization of nutrients from senescent leaves. Studies have revealed that any manipulation enabling a delay of senescence might affect mineral content of grains. Down-regulation of a NAC transcription factor, NAM-BI resulted in delayed senescence, decreased gain protein and lowered iron and Zn concentration due to reduced nutrient remobilization from vegetative tissues [63].

The maize kernel consists of three main parts: embryo (composed of central embryo axis and the scutellum), endosperm and the pericarp [64]. In kernels, the highest concentrations of Zn colocalize with protein and free amino acids in the embryo with lower concentrations in endosperm [65,66]. However, approximately 90% of Zn in the embryo is present as Zn-phytate while in the endosperm, Zn is primarily complexed with an N-containing ligand such as histidine and to a lesser extent with phytate [65]. Thus, the Zn obtained from consumption of the endosperm is likely to be more bioavailable compared with Zn from embryo.

Phytoavailability, the transfer of trace elements from soil to the plants depends on soil properties, climatic conditions, and agronomic practices [67]. Soil properties that influence Zn availability include soil pH, organic matter content, cation exchange capacity, soil microorganisms, and clay concentration [48,68,69]. In alkaline soils, the availability of Zn is reduced [70]. Adding organic matter to the soil may increase the availability of Zn in the soil. Organic materials not only release free Zn into soil solution, but also changes the original solubility and mobilization of soil Zn by the formation of Zn organic complexes [71]. Finer texture soils like clay have a higher cation exchange capacity and therefore, have highly reactive sites and can retain more Zn than lighter textured soils such as sandy soils [72].

In plants, Zn deficiency is commonly associated with a reduction in yield and quality of produce [73]. Thus, diagnosis of a plant's Zn status is important. Initial symptoms of Zn deficiency appear on young leaves and meristems of maize plants due to the low mobility of Zn [74]. Such symptoms include leaf chlorosis, decrease in leaf size which in turn causes stunting and a decrease in the number of tillers [48,75]. Zn deficiency also decreases pollen viability in maize. Studies have shown that sub-normal supplies of Zn to maize plants at any stage of anther development induces male sterility [76].

Zn deficiency can be amended using soil-applied fertilizers or foliar sprays containing Zn. The highest values of Zn concentration in plants have been observed when Zn is applied via soil [77]. For maize, Zn-sulphate can be applied to the soil during planting. However, if Zn deficiency symptoms are observed late in the growing season, Zn sulphate can be applied as a foliar spray [78]. Possibly, crop yields in Zn-deficient soils may be improved by exploiting genotypic differences in Zn uptake and tissue use efficiency that exists within crop species [79–81].

The Genetics of kernel Zn accumulation in maize

Investigations on the genetics of kernel Zn content of maize were first reported in the 1960s and 1970s [82,83]. Additive gene action was reported to be more important than non-additive gene action for kernel Zn concentration [82,84–86]. In the 2000s, subsequent studies were focused on germplasm assessment in relation to the genetic potential for increasing the concentration of Zn in the grains of maize [12,86–89]. Several quantitative trait loci (QTL) mapping experiments have provided more information on the genetics of kernel Zn accumulation in maize [90–92].

Knowledge about the combining ability is essential in developing the best breeding strategy for utilizing the available germplasm. General combining ability (GCA) effects for

kernel Zn concentration were reported to be more important than specific combining ability (SCA) effects [82,84–86]. GCA is the average performance of a line in a hybrid combination whereas; SCA designates cases in which certain combinations perform relatively better or worse than would be expected on the basis of the average performance of the lines involved [93]. Significant GCA effects indicate that additive gene action is important in the inheritance of kernel Zn and genetic gains can be realized from selection.

To improve the concentration of Zn in maize kernels, it is essential to know the extent to which kernel Zn is heritable. Heritability estimate is a key parameter in determining response to selection for any target trait [94].

In maize, heritability ranging from 59-76% for kernel Zn have been reported [88,90,92,95,96]. The extent of heritability estimates reported suggests considerable influence of genetic factors in determining Zn in maize kernels.

The extent of interaction between genotypes with environments is very important in understanding the genetics of kernel Zn accumulation. Different studies have reported the existence of genotype by environment (G x E) interactions for kernel Zn concentration is an indication that maize genotypes may perform differently under different growing conditions [12,86,87,92,97,98]. Despite the existence of G × E interactions, a major proportion of the variation for Zn concentration is governed by genetic factors [87,97]. Therefore, it is feasible to identify and develop genotypes with increased kernel Zn concentration [99].

Understanding the genetic correlation among Zn, grain yield and with the other micronutrients such as iron (Fe) would facilitate the selection and breeding of Zn-dense maize genotypes. Presence of a strong and positive correlation will allow breeders to improve the correlated traits simultaneously. However, different magnitudes of correlations have been

reported. Lack of correlation between kernel Zn concentration and grain yield has been reported suggesting that improvement of Zn is possible without reducing the yield [12,98,100]. In contrast, negative correlation between grain yield and kernel Zn has been reported [12,90]. The negative correlation resulted in low-yielding varieties but with high Zn concentration. This may be due to a dilution effect whereby in the high-yielding genotypes, increased carbohydrate content in the grain possibly dilutes the concentration of Zn [12].

Kernel Zn concentration is positively correlated with kernel Fe concentration [84,90,92,98,103–105]. The positive correlation between Zn and Fe could possibly be due to (i) the linkage between the genes affecting the accumulation of these two micronutrients or (ii) commonly regulated mechanisms such as uptake and transport in the maize plant since some of the genes that encode metal transporter proteins transport multiple metals [92]. For example, IRT1, an *Arabidopsis* transporter important for Fe uptake can also facilitate the accumulation of Zn and Mn. In contrast, weak and no correlations between kernel Zn and Fe have been reported suggesting involvement of different genes in the accumulation of Zn and Fe [83,88,99]. Therefore, genetic improvement for Zn and Fe could be undertaken independently.

Detection and analysis of quantitative trait loci (QTL) has been proposed as an effective approach to further understand the genetic basis of kernel Zn accumulation [106]. Consistent with earlier studies (based on mating designs), additive gene effects predominantly controlled kernel Zn concentration [91,106]. Additionally, QTLs with additive effects, partially dominant and overdominant effects for kernel Zn concentration were reported [92]. QTL studies have also identified some of the possible reasons behind the correlation between Zn and other micronutrients such as Fe. The QTLs for Zn and Fe were co-localized on chromosomes 2 and 9 suggesting a possible reason for the correlation observed between Zn and Fe [92,107,108].

These co-localized QTLs may be pleiotropic in controlling the network of Zn and Fe uptake, transportation, and sequestration [109].

However, results from the several QTL studies conducted in maize (Table 3.1**)** were not consistent regarding genomic locations, total variance explained by the detected QTL and confidence intervals. The differences could possibly be attributed to the different genetic backgrounds (genotypes, populations and generations) of the populations used in each study, different environments under which the mapping populations were phenotyped, the different methods used to estimate the QTL effects, map density and population sizes [110–112]. So, it may be necessary to perform more QTL analyses for kernel Zn concentration to detect more loci and possibly identify consistent QTLs.

To obtain consistent QTLs, a comprehensive comparison among QTLs reported in independent studies may be helpful. QTL meta-analysis (MQTL) is an approach to integrate and comprehensively compare QTLs reported in independent studies so as to determine QTLs with more accurate position and smaller confidence intervals [113,114]. MQTL could increase the accuracy, provide more information on the genetic architecture of kernel Zn and enhance the speed of genetically improving kernel Zn concentration. MQTL has been used in different plant species to analyze various traits, for example, grain yield and related traits, flowering time and photoperiod sensitivity, drought tolerance, disease resistance, kernel Zn and Fe concentration [110,115–117]. Forty QTLs responsible for Zn concentration found in four studies were synthesized into nine MQTLs. Of the nine, two were identified in two genomic regions located on chromosome 2, a region that could be important for kernel Zn. These regions may be valuable for fine-mapping and map-based cloning [106].

MQTLs would also be helpful for maize improvement because the identified MQTLs could be selected through MAS to improve kernel Zn concentration.

Table 1.3 Summary of QTL studies conducted for Zn in maize

Parents	Mapping population	QTLS detected	Chromosome	Phenotypic variation explained (PEV)	Reference
B84 x Os6-2	F4	4	4	7.80%	[91]
Mu6 x SDM & Mo17 x SDM	F2:3	14	1,2,6,7,9,10	6.3-21.3%	[92]
B73 x Mo17	IBM	3	1, 4 and 5	5-10%	[90]
DH8 x DH40 & DH86 x S137	DH(a) & DH(b)	17	2, 3, 4, 6, 7 and 10	9.4-48.8%	[118]
178 x P53	F2:3	4	2,5,10	5.9-17.6%	[106]
178 x P53	RILS	20	4 and 5	2.7-16.8%	[119]

Comparative mapping of MQTL for kernel Zn can also be useful for revealing conserved syntenic relationships of Zn concentration among distinct species. Based on the analyses of MQTL controlling kernel Zn concentration in maize and rice, comparative mapping combined with homology-based cloning can help identify candidate genes in maize [110]. This is because genomic regions controlling the accumulation of Zn in maize kernels and rice grains have been reported to derive from a single ancestral genomic region.

Four MQTL associated with Zn accumulation in maize and rice were reported [110]. The MQTLS were on maize chromosome 2 co-linear with rice chromosome 7, maize chromosome 3 co-linear with rice chromosome 1, maize chromosome 5 co-linear with rice chromosome 2 and maize chromosome 9 co-linear with rice chromosome 3. A probe linking 2 MQTLs for maize and rice (syntenic MQTL-related regions) anchored onto metal transport protein-coding genes in maize and rice. This implied that; (i) kernel Zn concentration is syntenic between maize and rice (ii) and that the syntenic MQTL-related regions can be reliable for subsequent analysis like fine-

mapping and map-based cloning. Homology-based cloning using MQTL for kernel Zn concentration in maize and rice MQTL identified one candidate gene for Zn concentration in maize [110].

Results from previous studies have reported that some Quality Protein Maize (QPM) have a higher concentration of kernel Zn [84,98,120–123]. In a study comparing normal maize and QPM, some maize inbreds with the *o2* gene had higher Zn concentration [122]. Those QPM inbreds namely B8-*o2*, W64A-*o2* and OH51A-*o2* accumulated 16-35% more Zn than the corresponding non-QPM versions (B8, W64A and OH51A). Results from experiments conducted by CIMMYT have also shown that Zn concentration is high in QPM than non-QPM, although not all QPM is high in Zn (Dr. Thanda Dhliwayo personal communication). Increased levels of Zn concentration in QPM could be attributed to the direct influence of *o2* locus and other closely linked genetic factors [84]. In QPM, the presence of *o2* allele has also been reported to partially inhibit the synthesis of zein proteins, with a proportionate increase of globulins, glutelins and albumins, which are known to bind Zn in the endosperm of maize [124].

Conventional breeding to increase kernel Zn concentration in maize

Genetic biofortification aims to develop maize varieties with enhanced Zn concentration. The genetic biofortification process entails screening germplasm for available genetic diversity, identifying and testing Zn-enriched germplasm, conducting genetic studies and developing knowledge-based strategies (e.g. marker-aided selection) to lower the costs while accelerating the process of breeding. The Zn-biofortified varieties are tested in multiple locations and years to determine the genotype by environment interaction [15,22]. The Zn-enriched varieties should improve human nutrition without compromising yield and farmer-preferred agronomic traits

[15]. For instance, to guarantee widespread acceptance and use by farmers, the varieties should be high-yielding with adequate levels of resistance to biotic and abiotic stresses.

Nutritional breeding targets for Zn were established based on amount of maize consumed (g d^{-1}), bioavailability (% Zn absorbed), retention after processing (e.g. milling, storage and cooking) and the percentage of the daily requirement of Zn that should be obtained from maize [22,125]. For maize, the minimum target level of Zn is intended to provide at least 50% of the daily physiological requirement assuming an absorption and retention rates of 25% and 80%, respectively, upon consuming ~300g of uncooked maize per day. Based on this information, the target for Zn in maize was set at ~33 μg/g (Dr. Erick Boy personal communication). The average Zn concentration in maize ranges between 20-25 μg/g [125,126]. Thus, an increase of 8-13 mg/kg of Zn is achievable through conventional breeding, especially because a wide range of Zn concentration is available within maize germplasm.

Evaluating diverse germplasm for the presence of genetic variability is essentially the first step while breeding maize for improved Zn concentration [29,105]. Several studies have reported the existence of wide genetic variation for kernel Zn concentration (Table 1.4**)** suggesting the potential of genetically increasing Zn in maize. Objectives for exploring the available genetic diversity are to identify: (i) parental genotypes that can be used in crosses, genetic studies and molecular marker development, and (ii) existing varieties that combine high-Zn with desired agronomic traits for commercialization [15].

Besides measuring the extent of genetic variability, genetic and molecular analyses of Zn in relation to other components that potentially affect or contribute to kernel Zn concentration could be helpful [127]. This may be helpful for breeders especially while developing selection criteria for improving kernel Zn [98].

For instance, the positive and significant correlation for kernel Zn and kernel Fe concentration suggests the possibility of increasing Zn and Fe simultaneously [83–85].

Table 1.4 Genetic variability for kernel Zn concentration in selected studies since 2000

No.	Range Zn(μg/g)	Type of germplasm	No of germplasm	Place of evaluation	Reference
1	12.9-57.6	Landraces and improved genotypes	1,814	Zimbabwe & Mexico	[12]
2	11.65-95.6	Inbred lines	109	Nigeria	[104]
3	16.0-23.6	Hybrids	28	Croatia	[103]
4	16.5-20.5	Varieties	20	Nigeria	[87]
5	16.5-24.6	Varieties	49	Nigeria	[128]
6	19.4-24.6	Varieties	20	Nigeria	[129]
7	18.1-29.8	Inbreds	14	Zimbabwe	[86]
8	15.0-47.0	Core accessions	400	-	[11]
9	14.0-45.0	Inbreds	310	Nigeria	[105]
10	13.4-46.4	Inbreds	25	India	[98]
11	16.4-28.6	F_4 families	294	Croatia	[88]
12	17.6-49.1	Inbreds and hybrids	49	India	[127]
13	21.9-40.9	Inbreds	31	India	[130]
14	19.3-30.9	Hybrids	42	Mexico & Ethiopia	[101]
15	15.1-53.0	Inbreds and landraces	30	India	[102]
16	17.5-42.0	Inbreds	22	Brazil	[131]
17	7.0-29.9	Inbreds and landraces	67	India	[99]
18	3.8-35.8	Inbreds and landraces	81	India	[132]
19	12.6-39.4	QPM inbreds	46	India	[133]
20	5.4-30.8	Inbreds	50	India	[134]
21	19.4-32.6	Improved genotypes	48	India	[135]
22	20.0-53.0	Inbreds	24	Nigeria	[136]
23	17.1-43.8	Inbreds	923	Mexico	[123]

Combining both conventional breeding and agronomic strategies, breeders can effectively increase the levels of Zn in maize. Agronomic approaches includes fertilizer application by adding an appropriate mineral or inorganic compound to fertilizer [29,137]. The agronomic approach is the simplest method, but confounding effects can occur due to differences in mineral mobility, accumulation and different soil compositions in the specified geographical location, making a success of this method highly inconstant [138]. Conventional breeding which involves

identifying and developing inbreds with enhanced kernel Zn has also proven to be successful [139]. However, there are limitations with regard to the time needed to generate inbreds with enhanced levels of Zn concentration [140,141].

A continuous improvement of breeding strategies as well as the potential to use molecular techniques can enhance selection for Zn in maize kernels. For example, use of DNA-based markers can effectively complement phenotypic characterization and aid in the identification of promising lines that have high genetic diversity for breeding purposes [142]. Characterization of inbred lines using markers could also potentially aid in the selection of suitable lines for developing mapping populations and subsequently QTL analysis (White and Broadley, 2005; Chakraborti et al., 2011a; Sadeghzadeh, 2013).

Using genome-wide association studies (GWAS), the genetic architecture of kernel Zn in maize can be dissected allowing breeders to improve breeding efficiency by facilitating the introgression of related genes into low-Zn germplasm through marker-assisted selection or genomic selection (GS). Recently, genomic regions associated with kernel Zn concentration were identified in 923 inbreds adapted to the tropics [123]. The study reported 20 SNPs significantly associated with kernel Zn and 11 SNPs were validated in bi-parental populations. However, kernel Zn concentration is considerably influenced by environmental factors such that the identification and quantification of rare QTLs with small phenotypic effects may not be realistic [145]. GS uses genome-wide genetic information to predict genetic values of candidates genotypes. In GS, molecular and phenotypic data are combined in a training population to estimate genomic estimated breeding values of genotypes in a breeding population that has been genotyped but not phenotyped [146].

Preliminary genomic prediction results suggest that GS could potentially capture both major and minor effect QTL for improving kernel Zn concentration [147]. Relative to conventional phenotype-based selection, GS can accelerate genetic gains in the development of Zn-enriched maize varieties through early selection. Kernel Zn concentration is determined late in the development stage of a maize plant (after physiological maturity). If GS models are applied to select potential candidates in the early stages of development, they could reduce the resource- and time intensive process of field evaluation for kernel Zn concentration. Therefore, the development of Zn-dense maize varieties through conventional methods and the use of markers can be cost-effective, sustainable and realistic.

Transgenic breeding for increased Zn concentration in maize

Transgenic plant breeding could be a promising approach in efforts to produce biofortified crop varieties with desired levels of micronutrients. This strategy is more applicable in crops in which the targeted micronutrient does not naturally exist at the required level among the many varieties in a germplasm [15]. The transgenic strategy differs from the conventional approach in that new ‘genetic factors’ may be introduced into the plant’s genome. The new ‘genetic factors’ are introduced into the genome in some forms of transgenics but in others, such as CRISPR-Cas, segregation eliminates the ‘transgenes’ and leaves behind the modified genome. The transgenic breeding approach aims at enhancing Zn content by: (i) increasing the efficiency of uptake and transport of Zn to the harvestable tissue, (ii) increasing the amount of bioavailable Zn accumulating in the plant [144], and (iii) reducing the antinutritional factors in the edible part and increasing the promoters for example promoters of Zn bio-availability in maize kernels [148].

Progress has been made in developing transgenic plant genotypes with increased concentrations of micronutrients. For example, transgenic rice that can provide 30% of the daily physiological requirement for both Zn and Fe has been developed and tested in confined field trials [149]. Golden rice, a transgenic rice variety high in β-carotene can provide more than 50% of the daily physiological requirement for vitamin A [137,150]. A transgenic bio-fortified maize expressing high amounts of β-carotene, ascorbate, and folate has also been developed [151]. Relative to kernels from conventionally-bred maize, the transgenic maize kernels contained 169-fold the normal amount of β-carotene, 6-fold the normal amount of ascorbate, and double the normal amount of folate suggesting the possibility of developing maize high in kernel Zn through transgenic breeding [152].

The transgenic approach in breeding can also serve as one of the potential tools in breeding maize varieties with low kernel phytic acid. Phytic acid is known as a major source of phosphorous in cereal grains. During the process of seed germination, phytase is activated to degrade phytate, releasing the stored phosphorus to be utilized by the developing seedling [153]. Phytate has six negatively charged ions making it a strong chelator of Zn cations. In view of this, phytic acid is considered one of the potential antinutritional factors hindering absorption of Zn by the digestive system [154]. In maize, more than 20 low-Phytic acid mutants have been isolated. These mutants resulted in a reduction of seed phytic acid up to 90% [155,156]. However, while breeding for low phytic acid, it is necessary to obtain plants that combine robust and agronomically desirable characteristics because this will highly contribute to farmers adopting the varieties.

Genome editing, a recent technology that gives scientists the ability to edit and change an organism’s DNA has been introduced. The technology allows genetic material to be added,

removed or altered at specific locations in the genome. The technology is a nuclease-based procedure of plant engineering using nucleases such as TALENS (transcription activator-like effector nucleases) or the CRISPR (clustered regularly interspaced short palindromic repeats). This technology can create precise insertions, deletions and substitutions in plant cells [157,158]. Genome editing may also help how breeders work in efforts to improve and enhance food crop nutritional status and quality. Using this technology, it is less likely that the new varieties will be subjected to the same strict set of regulations as are currently held for transgenic crops because segregation eliminates the 'transgenes' and leaves behind the modified genome [159].

Even though transgenic varieties have an incredible potential to improve the nutritional profile of maize kernels, their release to farmers may take too long because of strict release and approvals through national biosafety and regulatory processes [15]. While developing the transgenic varieties, reliance on random transgene insertion can be a challenge because of random genome integration. Random integration of transgene sequences into the genome may potentially disrupt endogenous gene expression in genic regions via insertional mutagenesis, thereby creating undesired side effects [160]. There is also a growing concern that introducing foreign genes into food plants may have an unexpected and negative impact on human health although there are no clear research results suggesting negative effects of these food plants. These negative aspects increases the lack of predictability in the performance of transgenically derived materials and further compromise the probability of successful product development, deployment and adoption [161].

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CHAPTER 2. A GENETIC EVALUATION OF KERNEL ZINC IN HYBRIDS OF ELITE QUALITY PROTEIN MAIZE (QPM) AND NON-QPM INBRED LINES ADAPTED TO THE TROPICS

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Abstract

Genetic improvement of maize with elevated levels of zinc (Zn) can reduce Zn deficiency among populations who rely on maize as a staple. Inbred lines of quality protein maize (QPM) and non-QPM with elevated Zn levels in the kernel have been identified. However, information about the optimal strategy to utilize the germplasm in breeding for high-Zn concentration is lacking. As a preliminary step, this study was conducted to ascertain the potential of QPM, non-QPM or a combination of QPM and non-QPM hybrids for attaining desirable Zn concentration. Twenty elite inbreds, 10 QPM and 10 non-QPM, were crossed according to a modified mating design to generate hybrids which were evaluated in four environments in Mexico during 2015 and 2016. Results indicated the importance of assessing the genetic potential of inbreds to serve as parents for Zn-biofortified hybrids on the basis of their hybrid progenies. The highest mean values of Zn were observed when high-Zn QPM lines were crossed to high-Zn non-QPM lines.

Hybrids with high Zn and grain yield were identified. General combining ability (GCA) effects for Zn concentration were more preponderant than specific combining ability (SCA) effects, suggesting the importance of additive gene action for the inheritance of Zn.

Keywords: Genetics; maize; zinc; QPM; kernels; combining ability; breeding

Introduction

Micronutrient deficiency, resulting from inadequate intake of essential minerals such as zinc (Zn), is an increasingly serious food-related health problem [1]. Approximately 20% of the world's population suffers from Zn deficiency with the highest risks for young children and pregnant women in sub-Saharan Africa and south Asia [2]. Approaches to mitigate Zn deficiency include diet supplementation, industrial fortification and food diversification. However, on a large-scale, the impact of these interventions remains limited especially in low-income countries due to recurrent costs, poor infrastructure and delivery systems [3,4]. Therefore, development of Zn-enriched staple crops through breeding may complement those options [5–7].

Maize is one of the major crops grown and consumed in regions where Zn deficiency is prevalent [8–10]. For instance, in sub-Saharan Africa, 80% of the maize is consumed directly as food, providing at least 30% of the total calories [10–12]. However, maize improvement programs have primarily focused on developing high-yielding varieties able to tolerate various biotic and abiotic stress factors in different agro-ecologies [13]. Therefore, the production of micronutrient-rich varieties has lagged behind the improvement of other traits.

The physiological processes by which Zn accumulates in the maize kernels have not been completely described. A maize plant acquires Zn through the roots with uptake mediated by Zn-regulated transporters [14].

Then, Zn is transferred to the vascular bundles for transport to the shoot [15] and remobilized from the leaves into the kernels during grain-filling [16]. In kernels, higher concentrations of Zn are observed in the aleurone and embryo than in the endosperm [17,18].

The plant's ability to accumulate Zn in the kernels can also be influenced by environmental conditions and soil properties. For example, an increase in soil pH decreases the uptake of Zn from the soil and reduces its availability to the plant [19,20]. Low soil moisture, organic matter content and temperature impairs Zn diffusion to the roots causing reductions in uptake and translocation into the shoot [21–23]. Consequently, the genetic capacity of a plant to absorb Zn from the soil and accumulate it in the kernels for optimal nutritional benefit may not be fully realized.

The successful identification of desirable hybrid combinations depends on the combining ability of the parents and the gene effects involved in the expression of a trait. Several genetic studies involving mating designs documented that for kernel Zn general combining ability (GCA) effects were greater than specific combining ability (SCA) [24–27]. Significant GCA effects indicate the preponderance of additive gene action for kernel Zn inheritance, implying that genetic gains can be realized from selection.

Kernel Zn has been investigated in several analyses of quantitative trait loci (QTL) which have shown that Zn accumulation is under the control of several loci, from 4 to 20 per population [28–33]. Additionally, genomic regions associated with important QTLs for kernel Zn have been reported on chromosomes 2 and 6 [32,33]. Consistent with mating designs, additive gene effects predominantly controlled kernel Zn concentration in the QTL studies. The QTL studies, however, were conducted with populations of inbred progeny created from parents unadapted to

tropical environments [29–33]. So, the relevance of those studies for hybrid breeding for tropical environments may be limited.

In maize, nutritional-related research has emphasized on quality protein maize (QPM) to address protein malnutrition [34–36]. Inbred lines with high-Zn have been identified among QPM [37–43]. The high-Zn values suggests a possible influence of opaque2 (*o2*) locus or possibly other genetic factors present in the QPM lines [43]. However, QPM maize with relatively low levels of Zn have been observed suggesting that although *o2* may play an important role, there might be other favorable loci unrelated to *o2* that are required for the enhancement of Zn [18,38].

Significant differences in concentration of Zn have also been documented among non-QPM inbred lines [26,37,42,44–48]. The variability for kernel Zn among the inbred lines suggests a possibility to enhance the Zn content in maize [4]. In the present study, groups of QPM (high-Zn QPM and low-Zn non-QPM) and non-QPM (high-Zn non-QPM and low-Zn non-QPM) inbred lines adapted to tropical environments were mated to produce hybrids using a modified mating design. The objectives of this study were (i) to estimate the combining ability of elite QPM and non-QPM inbred lines for kernel Zn, (ii) to explore the potential of developing high-Zn hybrids using QPM, non-QPM and/or a combination of QPM and non-QPM inbred lines, (iii) to investigate the relationship between kernel Zn and other traits of agronomic importance and (iv) to evaluate the relative importance of additive and non-additive genetic effects for Zn

Materials and Methods

Ten Quality Protein Maize (QPM) and 10 non-QPM inbred lines adapted to the tropical and sub-tropical environments were selected for this study (Table 2.1). The inbred lines were

selected based on the Zn level in the kernel and whether classified as QPM or non QPM. The Zn levels of the inbreds were determined in evaluations in previous seasons in the same environments used for this study. Also, the lines were developed and selected based on their agronomic performance and potential to serve as parents of hybrids suitable for production in the target environments in central America and Mexico.

The lines were divided into four groups of five inbreds, according to their Zn levels and whether classified as QPM or non-QPM, based on their lysine and tryptophan content. The four groups were high-Zn QPM (>33 µg/g, Zn target level for nutritional impact; [51]), low-Zn QPM (<33 µg/g), low-Zn non-QPM and high- Zn non-QPM (Table 2.1). Intergroup crosses were made by mating each line from one group to the five lines in another group to form six sets of 25 hybrids (Table 2.2). Five out of the six sets produced enough kernels for evaluation in trials from all 25 expected hybrids per set. Two crosses between inbred lines 13 and 16 and 15 and 17 in set six did not produce enough kernels and the crosses were discarded. Therefore, set six produced 23 hybrids instead of 25. Kernels from reciprocal crosses in each set were bulked. In total, 148 hybrids were formed at Agua Fria, Puebla, Mexico, during the months of November 2014 through May of 2015.

The mating among the inbreds was intended to fulfill the requirements of a North Carolina design II [52]. However, mating among the lines in each set was slightly different from the standard design II [52], since each inbred line was not used strictly as female or male (Table 2.2). All inbred lines were used multiple times (as females, males or both) to form hybrids in different sets (Table 2.1).

Table 2.1 Maize inbred lines used as parents for the mating design and their Zn levels

Line	Pedigree	Group	Description	Role of inbred line	Set
1	((CML491/LAPOSTASEQ-C7-F64-2-6-2-2-B*3)/CML491)-B-7-1-1-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
2	((CML491/LAPOSTASEQ-C7-F64-2-6-2-2-B*3)/CML491)-B-37-1-1-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
3	((CML491/((((CML176/R1)/R1)-73-1-3-1/R1)-79/R1)-36-1-B)/CML491)-B-18-1-1-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
4	((CML491/LAPOSTASEQ-C7-F103-2-2-2-1-B*3)/CML491)-B-17-2-1-1-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
5	((CML491/LAPOSTASEQ-C7-F64-2-6-2-1-B-B)/CML491)-B-50-1-2-1-1-B	A	High Zn, QPM	Female	1, 2 & 3
6	((CML491/CML150)/CML491)-B-13-1-1-1-1-1-B-B	B	Low Zn, QPM	Male & Female	1, 4 & 5
7	((CML491/CML150)/CML491)-B-21-1-1-1-1-B-B	B	Low Zn, QPM	Male & Female	1, 4 & 5
8	((CML491/LAPOSTASEQ-C7-F64-2-6-2-1-B-B)/CML491)-B-30-1-1-1-1-B-B	B	Low Zn, QPM	Male & Female	1, 4 & 5
9	CML247Q	B	Low Zn, QPM	Male & Female	1, 4 & 5
10	CML254Q	B	Low Zn, QPM	Male & Female	1, 4 & 5
11	(CML550/CML511)-B-62-2-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
12	(CLG2312/CML9)-B-80-1-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
13	(CML550/CML511)-B-106-1-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
14	((CRIOLLOTTH/CML247)/CLRCW105)-B-37-1-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
15	(CLG2312/CML505)-B-43-1-1-1-B	C	Low Zn, Non-QPM	Male & Female	2, 4 & 6
16	((CML247/((((CML176/R1)/R1)-73-1-3-1/R1)-79/R1)-36-1-B)/CML247)-B-14-2-1-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6
17	((CML247/((((CML176/R1)/R1)-73-1-3-1/R1)-79/R1)-36-1-B)/CML247)-B-18-1-1-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6
18	(CLRCW79/CLRCW98)-B-14-2-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6
19	(CLRCW79/CLRCW98)-B-16-2-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6
20	(CLRCW79/CLRCW98)-B-22-3-1-1-B-B	D	High Zn, Non-QPM	Male	3, 5 & 6

Table 2.2 Mating scheme used to form hybrids

			Males																		
	Inbred lines		1	2	3	4	5	6*	7*	8*	9*	10*	11*	12*	13*	14*	15*	16	17	18	19	20
			Group 1					Group 2					Group 3					Group 4				
Females	1	Group 1						1	1	1	1	1	2	2	2	2	2	3	3	3	3	3
	2							1	1	1	1	1	2	2	2	2	2	3	3	3	3	3
	3							1	1	1	1	1	2	2	2	2	2	3	3	3	3	3
	4							1	1	1	1	1	2	2	2	2	2	3	3	3	3	3
	5							1	1	1	1	1	2	2	2	2	2	3	3	3	3	3
	6*	Group 2											4	4	4	4	4	5	5	5	5	5
	7*												4	4	4	4	4	5	5	5	5	5
	8*												4	4	4	4	4	5	5	5	5	5
	9*												4	4	4	4	4	5	5	5	5	5
	10*												4	4	4	4	4	5	5	5	5	5
	11*	Group 3																6	6	6	6	6
	12*																	6	6	6	6	6
	13*																	6	6	6	6	6
	14*																	6	6	6	6	6
	15*																	6	6	6	6	6

The numbers within the table indicate the sets formed by mating inbred lines designated as females and males
Group 1=high-Zn QPM; Group 2=low-Zn QPM; Group 3= high-Zn non-QPM; Group 4 = low-Zn non-QPM
Inbred lines 1-5 were used as Females to form hybrids sets denoted with numbers 1, 2 and 3. Inbred lines with an asterisk (*) were used both as female and male to form hybrid sets denoted with numbers 1, 2, 5, and 5 and inbred lines 16-20 were used as males to form hybrids sets denoted with numbers 3, 5 and 6.

As a consequence, variance components (σ_A and σ_D) could not be estimated since: (i) the inbred lines were selections made from a breeding program, and not a random sample from a population, (ii) for each set, the sample size was small (10 inbred lines per set) and (iii) data could not be pooled across sets to estimate the sum of squares because the same inbred lines were used to form hybrids in different sets. Hence, analyses were conducted within sets to calculate means for the hybrids and estimates of GCA and SCA effects of inbred lines and hybrids, respectively. These parameters are not influenced by the variance components.

One hundred and forty-eight hybrids plus two commercial hybrid checks were grown in four environments at CIMMYT's and INIFAP (National Agricultural Research Institute) research stations in Mexico, during the months of June through October. The four environments were Tlaltizapan (18°41' N, 99° 07' W; 962.5 meters above sea level [m asl]) during 2015 and 2016, Agua Fria (20°32´ N, 97°28´ W, 110 m asl) during 2015 and Cotaxtla (18°49' N, 96° 22' W,57 masl) during 2015. The experimental design was an alpha-lattice [50] using two replications and one-row plots. All plots were managed according to the recommended agronomic practices for each environment.

At Tlaltizapan, the experimental unit was a 5-m-long plot, with an inter-row spacing of 0.75 m and a spacing of 0.14 m between hills, giving a final plant density of approximately 93,000 plants ha^{-1}. At Agua Fria, the experimental unit was a 4.5-m-long plot, with an inter-row spacing of 0.75 m and a spacing of 0.30 m between hills, giving a final plant density of approximately 44,444 plants ha^{-1} and at Cotaxtla the experimental unit was a 5-m-long plot, with an inter-row spacing of 0.80 m and a spacing of 0.20 m between hills, giving a final plant density of approximately 63,000 plants ha^{-1}.

Data were recorded on a plot basis on several traits in each experiment: days to anthesis days to silking, anthesis silking interval (ASI), plant height, grain yield and kernel Zn concentration. Days to anthesis was recorded as the number of days from planting to when 50% of the plants in a plot were shedding pollen, and days to silking was the number of days from planting to when 50% of the plants in a plot had extruded silks. ASI was determined as the difference between days to silking and days to anthesis. Plant height was measured in centimeters as the distance from the base of the plant to the top of the first tassel branch. Grain yield, expressed in tons ha^{-1} was determined by adjusting the shelling and grain moisture percentages to 80 and 12.5, respectively. Shelling percentage, calculated as ((grain weight/ear weight) * 100), was used to determine the grain weight in a plot. Normally, at harvest the shelling percentage averages about 80% [51]. Grain moisture at harvest was measured using a hand-held moisture meter. Samples for grain moisture content were obtained by removing several rows of maize kernels from 10 randomly selected ears per plot/row.

The following formula was used to estimate grain yield in ton ha^{-1}:

$$\frac{field\ weight\ in\ kgs}{1000} * \frac{100 - moisture\ content\ \ per\ plot}{100 - 12.5} * \frac{10000}{plot\ area} * shelling\ percentage$$

In each plot of all environments, four to six plants were self-pollinated and harvested with husk for the determination of kernel Zn concentration. These ears were not used to estimate grain yield.

At physiological maturity, the self-pollinated ears from each plot were manually harvested and dried to a moisture content of 12.5 %. Kernels from the ears were hand-shelled, bulked and a representative sample was obtained from each plot. Approximately, six grams of kernels from each plot were ground to a fine powder (flour approx. 0.5 µm) using a

Retsch™miller (model MM400) and a 35mL grinding milling jar of zirconium. Flour was collected in 15 mL plastic tubes for Zn content analysis using a 'bench-top', non-destructive, energy-dispersive X-ray fluorometer (XRF) Oxford instruments ™, model X-Supreme 8000 ®. Five grams of flour were placed into the polypropylene capsules and sealed with a Poly-4 ® XRF film for scanning. Briefly, before the samples were analyzed, the equipment was calibrated by relating the X-ray emission intensity of Zn to a group of samples whose Zn concentration had been previously determined through inductively coupled plasma optical emission spectrophotometer (ICP-OES). The calibrations were validated by comparing the values given by the XRF with those from the ICP-OES. This was done using a group of samples different from the ones used in the calibration. To confirm the values obtained by XRF, 10% of the samples were re-analyzed by the ICP-OES [52]. In the ICP-OES analysis, aluminum was also monitored as indicators of contamination [38].

The trials were analyzed according to an alpha-lattice design using multi-environmental trial analysis with R (META-R) [53]. Variance components due to genotypes $(\sigma^2 G)$ genotypes by environment $(\sigma^2 G \times E)$ interactions and residual errors $(\sigma^2 e)$ were estimated from the analysis of variance (ANOVA). Replications and incomplete blocks within replications were considered random effects while genotypes (hybrids) and environments were considered fixed effects. Genotypes were considered as fixed effects because they were developed from inbred lines that were specifically selected from a breeding program, had different levels of kernel Zn concentration and were classified as QPM or non-QPM. Hence, inference is limited to the population from which the inbred line was selected from and to the environments under which the hybrids were evaluated. Broad-sense heritability (H^2) for traits in individual and across environments was estimated using the variance components [54].

To estimate combining ability of the inbred lines, a separate analysis of variance was conducted for the 148 hybrids (excluding the checks), according to a modified mating design using the proc GLM statement of SAS [55]. Hybrids were nested within sets for each environment and across environments. Components of variance due to hybrids within sets were divided into variance due to female (sets), male (sets), and the interaction between female x male (sets). The *F* tests for female (sets), male (sets), and female x male (sets) mean squares were computed using the mean squares for their respective interaction with environment. Mean squares attributable to female (sets) x environment, and male (sets) x environment were tested using the mean square for female x male (sets) x environment whereas the mean square for female x male (sets) x environment was tested using the pooled error mean squares. The expectations of females (set) and males (set) represents the general combining ability (GCA_f and GCA_m) effects, while the interaction between female x male (set) represents specific combining ability (SCA) effects [54]. The proc mixed statement of SAS [55] was used to calculate adjusted means for grain yield and kernel Zn in individual sets. Sets were considered fixed effects: thus, their interpretation was based on means and differences and inferences were limited only to the specified set [56]. The allocation of inbred lines as males or females was random. Therefore, males and females within sets, interaction of female and male within set and all their interactions with the environment were considered random effects. For random effects, the measure of interest is the variance [56], and inferences can be made relative to the reference population and how they interact with the environments [54]. Estimates of GCA effects for kernel Zn, grain yield and days to flowering for the inbred line were calculated in each environment and across environments. For kernel Zn concentration, the SCA effects for each cross over environments were also estimated.

Although mating among the lines deviated from a standard NC II, the expectations of the mean squares for females (set), males (set) and females x males (set) were the same for the components of variance and the covariances of relatives as in a standard NC II [54]. Assuming no epistasis and a coefficient of inbreeding of one, variance components can be expressed in terms of covariance (Cov) of relatives where $\sigma^2_{male} = \sigma^2_{female}$ = Cov half-sib = (1/2) σ^2_A, and $\sigma^2_{male\ x\ female}$ = Cov full-sib - Cov half-sib male - Cov half-sib female = σ^2_D [57]. Therefore, the variance explained by the GCA effects of parents = 1/2 additive genetic variance [VA] and the variance explained by SCA = dominance genetic variance [VD]. The relative contribution of GCA (additive) and SCA (non-additive) genetic variances for kernel Zn in each set were computed relative to the total genetic variance.

Results

Genotypes (hybrids) and genotype x environment (G x E) variance components were significantly different ($p < 0.001$) for Zn and grain yield (Table 2.3). For Zn, the variance component for genotypes was larger (~3-fold) compared to the variance due to G x E and the heritability (H^2) estimate was 0.85. For grain yield, the variance due to genotypes was 5-fold larger than the G x E variance component and H^2 estimate was 0.91.

Averages for Zn and grain yield of all hybrids for each environment and across environments were estimated (Table 2.3). The highest mean for Zn (26.51 µg/g) was observed in Tlaltizapan 2016 and lowest was in Tlaltizapan 2015 (22.47 µg/g). Tlaltizapan 2016 was an exceptional environment in which 21 hybrids accumulated ≥ 30 µg/g of Zn (Table A.1). The mean grain yield across environments was 7.07 t ha^{-1} with range of 8.75 t ha^{-1} for Tlaltizapan 2015 to 4.87 t ha^{-1} for Cotaxtla 2015 (Table 2.3).

Table 2.3 Performance of the top ranking 10% hybrids for Zn and their grain yield, averages, heritabilities and variance components at each environment and across environments

Hybrid	Cross	Group	Tlaltizapan 2015		Tlaltizapan 2016		Agua Fria 2015		Cotaxtla 2015		Average across environments	
			GY	Zn	GY	Zn	GY	Zn	GY	Zn	GY	Zn
			t ha^{-1}	µg/g	t ha^{-1}	µg/g	t ha^{-1}	µg/g	t ha^{-1}	µg/g	t ha^{-1}	µg/g
56	2 x 16	A X D	10.35	28.32	9.14	28.96	6.04	32.94	6.79	32.58	8.22	31.45
51	1 x 16	A X D	9.73	26.78	11.07	31.35	7.74	33.33	5.8	30.46	8.8	31.07
60	2 x 20	A X D	11.18	25.8	9.51	32.76	8.93	35.38	7.31	25.79	9.4	30.25
57	2 x 17	A X D	9.8	25.27	10.12	30.2	5.92	31.9	4.71	28.21	7.8	29.26
55	1 x 20	A X D	10.01	25.39	11.24	29.69	7.7	32.8	4.9	25.61	8.61	29.07
1	1 x 6	A X B	6.2	26.85	6.34	30.1	2.88	31.34	2.31	24.22	4.11	28.72
21	5 x 6	A X B	3.84	27.83	4.72	31.5	1.79	26.43	1.37	-	2.48	28.66
23	5 x 8	A X B	5.95	26.06	5.6	32.25	4.6	25.42	2.91	29.16	4.62	28.62
66	4 x 16	A X D	10.4	25.54	8.74	27.84	6.81	31.99	6.65	27.39	8.15	28.61
11	3 x 6	A X B	5.82	23.88	4.65	32.53	2.36	28.51	1.77	-	3.23	28.28
65	3 x 20	A X D	10.2	24.07	10.93	27.6	6.7	31.95	4.8	27.87	8.29	28.16
13	3 x 8	A X B	4.33	24.41	3.56	28.76	1.2	31.74	1.9	25.27	2.33	27.79
69	4 x 19	A X D	10	26.33	10.98	30.56	7.77	26.87	4.74	26.01	8.49	27.78
125	10 x 20	B X D	7.73	24.3	6.63	27.95	4.49	28.75	3.53	28.36	5.38	27.76
7	2 x 7	A X B	5.12	26.4	5.8	28.62	4.12	25.93	3.34	27.78	4.38	27.71
Trial Mean			8.75	22.47	8.57	26.51	6.1	25.52	4.87	24.3	7.07	24.7
Mean top 15			8.04	25.82	7.94	30.04	5.27	30.35	4.19	27.59	6.29	28.88
Min			3.84	17.83	3.56	20.43	1.08	19.04	1.37	19.45	2.33	18.93
Max			12.59	28.32	11.24	32.77	8.93	35.38	7.31	32.58	9.4	31.45
LSD $_{0.05}$			1.4	2.68	1.61	2.9	1.12	2.97	1.43	3.27	1	0.87
Heritability			0.87	0.75	0.83	0.82	0.91	0.83	0.76	0.73	0.91	0.85
σ^2 G			3.76	7.33	3.64	11.09	3.33	12.91	2.15	8.81	2.79	7.59
σ^2 G x E			-	-	-	-	-	-	-	-	0.49	2.48
Residual			1.08	4.84	1.47	4.97	0.66	5.29	1.39	6.68	1.11	1.07

σ^2 G, Genotype variance and σ^2 G x E, the interaction between genotype and environment were significant at $\alpha = 0.001$. GY and Zn = Grain yield and Kernel zinc concentration, respectively. The LSDs are for comparing the means among hybrids. Group A, B and D =high Zn QPM line, low Zn non-QPM line and high Zn non-QPM line, respectively.

The Pearson correlation coefficient values between pairs of traits ranged from -0.09 for Zn and plant height to 0.20 between Zn and days to anthesis (Table S3). Correlation values between Zn and flowering dates (anthesis and silking date) were low (0.16-0.20) but significantly different from zero at p-value <0.05 in each environment. Across environments, there was no significant correlation between Zn and any other trait.

The lack of an association between Zn and other traits is promising for maize breeding. Across environments, 15 hybrids were ranked as the top 10% for Zn (Table 2.3). Those hybrids involved at least one inbred from the high-Zn group (QPM or non-QPM), had 12-27% Zn content above mean of all hybrids in all environments (24.70 μg/g), and they were produced from thirteen inbred parents. Five inbreds were from the high-Zn QPM group and four inbred lines each were from the high-Zn non-QPM and low-Zn QPM groups. Six of the 15 hybrids were exclusively produced from QPM inbred lines, while nine were from crosses between QPM and non-QPM inbred lines. Inbred 2 from the high-Zn QPM group and 20 and from the high-Zn non-QPM group were parents to four hybrids each.

Among the top 10% hybrids with high-Zn across environments, high-yielding hybrids with 7.80-9.40 t ha^{-1} of grain were identified (Table 2.3). Inbred lines 1, 2, 3 and 4 from the high-Zn QPM group and 16, 17, 19 and 20 from the high-Zn non-QPM group were parents to those hybrids. However, despite the lack of correlation between grain yield and Zn, some of the hybrids which showed high-Zn concentration across environments were low-yielding. Overall, grain yield averages of the top 10% hybrids for Zn were 8-13% lower compared to the averages for all hybrids in each environment and across environments.

The average values of Zn for each inbred line as measured in their hybrids were estimated for each environment and across environments (Table 2.4). Based on those hybrids,

average values for Zn among the four groups of inbred lines (A: high-Zn QPM, B: low-Zn QPM, C: low-Zn non-QPM and D: high-Zn non-QPM) ranged from 21.15 to 27.97 μg/g. In all environments, the highest average value for Zn corresponded to high-Zn QPM inbreds (26.00 μg/g) while the lowest average value of Zn was recorded for low-Zn non-QPM inbreds (22.96 μg/g).

Table 2.4 Average kernel Zn and grain yield for inbred lines as observed in hybrid progenies

Inbred line	Group	Hybrid Zn levels (μg/g)					Hybrid GY across environments
		Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Across environments	t ha^{-1}
1	A	24.31 (15)	27.83 (15)	27.26(15)	25.10 (15)	26.30 (15)	7.16 (15)
2	A	24.43 (15)	29.09 (15)	27.91 (15)	26.01 (15)	27.08 (15)	7.59(15)
3	A	22.58 (15)	27.64 (15)	26.57(15)	24.78 (15)	25.44 (15)	7.32 (15)
4	A	23.15 (15)	27.45 (15)	26.35 (15)	24.59 (15)	25.43 (15)	7.36 (15)
5	A	24.23 (15)	27.83 (15)	25.53 (15)	24.83 (15)	25.77 (15)	7.12(15)
		23.74	**27.97**	**26.72**	**25.06**	**26**	**7.31**
6	B	22.90 (15)	28.23 (15)	26.44 (15)	24.48 (15)	25.64 (15)	5.53 (15)
7	B	22.73 (15)	25.78 (15)	25.40 (15)	24.63 (15)	24.67 (15)	5.54 (15)
8	B	23.56 (15)	27.30 (15)	26.16 (15)	25.48 (15)	25.73 (15)	6.61 (15)
9	B	22.21 (15)	25.53 (15)	25.45 (15)	24.79 (15)	24.48 (15)	6.74 (15)
10	B	22.44 (15)	28.41 (15)	25.51 (15)	24.75 (15)	25.27 (15)	6.61 (15)
		22.77	**27.05**	**25.79**	**24.83**	**25.16**	**6.21**
11	C	21.65 (15)	26.23 (15)	25.08 (15)	22.56 (15)	23.75 (15)	7.16 (15)
12	C	20.93 (15)	24.31 (15)	22.51 (15)	23.16 (15)	22.49 (15)	6.63 (15)
13	C	22.9 1(14)	27.25 (14)	25.81 (14)	24.79 (14)	25.21 (14)	7.99 (14)
14	C	20.35 (15)	22.83 (15)	22.97 (15)	22.78 (15)	21.94 (15)	6.63 (15)
15	C	19.94 (14)	23.15 (14)	21.86 (14)	22.00 (14)	21.41 (14)	7.38 (14)
		21.15	**24.75**	**23.64**	**23.06**	**22.96**	**7.16**

Table 2.4 continued.

Inbred line	Group	Hybrid Zn levels (µg/g)					Hybrid GY across environments
		Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Across environments	t ha^{-1}
16	D	23.93 (14)	27.46 (14)	27.99 (14)	26.99 (14)	26.52 (14)	7.30 (14)
17	D	22.49 (14)	26.63 (14)	25.95 (14)	24.96 (14)	25.02 (14)	6.67 (14)
18	D	21.41 (15)	26.64 (15)	25.41 (15)	24.83 (15)	24.53 (15)	7.68 (15)
19	D	22.67 (15)	26.52 (15)	25.31 (15)	24.23 (15)	24.66 (15)	7.31 (15)
20	D	22.93 (15)	27.23 (15)	28.51 (15)	25.01 (15)	25.98 (15)	7.72 (15)
		22.68	**26.9**	**26.63**	**25**	**25.34**	**7.34**
LSD $_{0.05}$		1.19	1.3	1.66	0.94	1.32	0.64

Hybrid Zn levels= average value of kernel Zn as observed in the hybrids that had a given inbred line as a parent. The number of hybrids evaluated for each inbred line is in parenthesis.
Hybrid GY (tons ha-1) = average value of GY as observed in the hybrids that had a given inbred line as a parent. The number of hybrids evaluated for each inbred line is in parenthesis.
The least significant difference (LSD) used for comparing the averages (in bold) among groups.

The genetic potential of the inbreds to serve as parents was assessed on the basis of their hybrid progenies (Table 2.4). The top five mean values for Zn in each environment and across environments involved inbred lines 1 and 2. The highest mean value for grain yield was observed for 14 hybrids which had inbred 13 as one of the parents. Genotypes with higher levels of Zn and grain yield were evident based on the performance of hybrids across the environments. Hybrids 51 and 60 were among the top ten hybrids with high Zn and grain yield (Table A.1). Based on the average grain yield for inbreds as assessed in hybrid combinations, inbreds 1, 2, 16 and 20, which were parents to hybrids 51 and 60 attained grain yields of ≥ 7 tons ha^{-1} (Table 2.4).

Analyses of means for Zn and grain yield were conducted for the hybrids across sets (Table 2.5). Values for average Zn ranged between 19.72 µg/g for low-Zn (QPM x non-QPM) hybrids to 29.69 µg/g for high-Zn (QPM x non-QPM) hybrids. The set of hybrids formed from high-Zn inbreds (QPM x non-QPM) had the highest mean for Zn while the set formed from low-Zn inbreds (QPM x non-QPM) attained the lowest mean for Zn.

Table 2.5 Averages for grain yield and Zn concentration for the sets of maize hybrids

Set composition	GY (t ha^{-1})					Zn (µg/g)				
	Tlalti 2015	Tlalti 2016	Agua Fria 2015	Cotaxtla 2015	Average	Tlalti 2015	Tlalti 2016	Agua Fria 2015	Cotaxtla 2015	Average
Group A x Group B	7.24	6.79	4.35	3.35	5.42	24.49	29.04	27.33	26.71	26.93
Group A x Group C	9.4	9.7	7.63	5.66	8.09	22.82	26.21	23.9	22.09	23.74
Group A x Group D	10.57	10.29	7.39	5.68	8.49	25.14	29.69	29.72	27.31	27.96
Group B x Group C	8.04	8.43	6.04	4.9	6.85	19.72	23.48	21.72	21.28	21.55
Group B x Group D	8.76	8.27	5.38	4.7	6.79	22.69	27.14	26.86	25.42	25.56
Group C x Group D	8.35	8.15	5.92	4.88	6.83	20.21	23.84	23.69	22.93	22.7
LSD $_{0.05}$	0.55	0.54	0.47	0.49	0.14	0.79	0.9	0.94	0.9	0.32

Values for grain yield (GY) and kernel Zn concentration (Zn) were significant at $\alpha = 0.001$ and 0.01.
Tlalti= Tlaltizapan. Group A, B, C and D = high-Zn QPM, low-Zn QPM, low-Zn non-QPM and high-Zn non-QPM, respectively. Each set consisted of 25 hybrids made by crossing each of five lines from one group to all five lines in the other group. The least significant difference (LSDs) are for comparing the averages among sets.

The average values for grain yield across the sets of hybrids ranged from 3.35 t ha^{-1} for high-Zn QPM x low-Zn non-QPM to 10.57 t ha^{-1} for high-Zn QPM x non-QPM hybrids (Table 2.5). Similarly, the set of hybrids formed from high-Zn inbreds (QPM x non-QPM) had the highest mean for grain yield while hybrids produced from QPM inbreds (high-Zn x low-Zn) had the lowest mean for grain yield.

Variances of general combining ability (GCA) effects, i.e. female, male, or both and specific combining ability (SCA) effects, i.e. female x male differed among the six sets of hybrids for all traits (Table 2.6). For Zn, significant variances due to GCA (female, male or both) were observed in five of the six sets. The SCA effects were significant only in set four (low-Zn QPM x non-QPM). Partitioning the variances in each set and across the four environments, GCA (GCA_m plus GCA_f) accounted for 76 to 96% of the variation observed in Zn (Table A.3).

Table 2.6 Analysis of variance of general combining ability (GCA) and specific combining ability (SCA) effects for grain yield and Zn concentration

Source of Variation	Set1	Set2	Set3	Set4	Set5	Set6
	Group A x Group B	Group A x Group C	Group A x Group D	Group B x Group C	Group B x Group D	Group C x Group D
Grain yield						
GCA_f	ns	ns	ns	**	***	***
GCA_m	***	ns	ns	*	ns	ns
SCA	***	ns	ns	***	***	*

Table 2.6 continued.

Source of Variation	Set1	Set2	Set3	Set4	Set5	Set6
	Group A x Group B	Group A x Group C	Group A x Group D	Group B x Group C	Group B x Group D	Group C x Group D
Zn concentration						
GCA_f	ns	***	*	ns	ns	***
GCA_m	*	**	*	***	ns	***
SCA	ns	ns	ns	*	ns	ns

*, ** and *** significant at p < 0.05, 0.01 and 0.001, respectively; ns =not significant (P >0.05)
GCAf = the general combining ability effect of the lines designated as females; GCAm = the general combining ability effect of the lines designated as males. Group A, B, C and D = high-Zn QPM, low-Zn QPM, low-Zn non-QPM and high-Zn non-QPM, respectively.

Six inbred lines showed positive GCA effects for Zn (Table 2.7). Among the six, two inbred lines, 2 and 8, were QPM and four, 11, 13, 16 and 20, were non-QPM. Inbred lines 1, 2, 16 and 20 were parents to hybrids that attained ≥30 mg/kg of Zn across environments (Table A.1). Inbred line 13 showed consistent, positive and significant GCA for Zn in each environment (Table A.4), and across environments (Table 2.7). This observation was not expected because that line was initially classified as a low Zn non-QPM. The significant and positive GCA effects indicated the inbreds would contribute favorable alleles for Zn in a breeding program if used as males or females, irrespectively. Inbred line 16 showed positive GCA for Zn and zero or negative GCA for grain yield (Table 2.7). Forty-one hybrids had positive SCA for kernel Zn (Table A.5).

Table 2.7 General combining ability (GCA) effects for grain yield and Zn across environments

Inbred line	Grain yield			Zn Concentration		
High Zn, QPM	LZn, QPM	LZn, non-QPM	HZn, non-QPM	LZn, QPM	LZn, non-QPM	HZn, non-QPM
1	0.00	-0.04	0.00	0.03	0.00	0.26
2	0.00	0.14	0.00	0.01	1.63**	1.18*
3	0.00	-0.03	0.00	0.02	-1.40*	-0.51
4	0.00	0.06	0.00	-0.05	0.03	-0.51
5	0.00	-0.13	0.00	0.00	-0.26	-0.42
Low Zn, QPM	HZn, QPM	LZn, non-QPM	HZn, non-QPM	HZn, QPM	LZn, non-QPM	HZn, non-QPM
6	-2.28*	0.23	1.16	0.51	0.00	0.02
7	-1.44	-0.14	0.33	-0.16	-0.63	-0.09
8	-0.51	0.67	1.28	0.28	0.48	0.13
9	1.80	-0.46	-0.45	-0.50	-0.10	-0.18
10	2.43*	-0.30	-2.31*	-0.13	0.25	0.12
Low Zn, non-QPM	HZn, QPM	LZn, QPM	HZn, non-QPM	HZn, QPM	LZn, QPM	HZn, non-QPM
11	0.00	0.00	0.26	0.44	0.68	1.01
12	0.00	0.00	-1.04*	-0.38	-0.27	-0.73
13	0.00	0.00	1.02*	2.34**	2.53**	2.53**
14	0.00	0.00	-0.71	-1.00	-1.16	-1.16
15	0.00	0.00	0.47	-1.40	-1.71*	-1.71
High Zn, non-QPM	HZn, QPM	LZn, QPM	LZn, non-QPM	HZn, QPM	LZn, QPM	LZn, non-QPM
16	-0.06	0.00	-0.08	1.55	0.34	1.43*
17	-0.50	0.00	-0.31	-0.63	-0.84	0.40
18	0.20	0.00	0.28	-1.01	0.13	-1.33*
19	0.05	0.00	-0.11	-0.59	-0.26	-0.95
20	0.32	0.00	0.23	0.73	0.63	0.45

* and ** Significant at p <0.05 and 0.01. Lines from one group were mated in a modified mating scheme to lines from three other groups so that three independent estimates of combining ability were computed for each line. LZn, QPM and LZn, non-QPM= low zinc QPM and non-QPM lines, respectively; HZn, QPM and HZn, non-QPM =high Zn QPM and non QPM lines, respectively.

Discussion

The inbreds' phenotype may provide useful information for creating hybrids with elevated levels of Zn in the kernel. In this study, hybrids with a Zn content ≥ 30 μg/g across environments were produced exclusively from inbred lines classified as high-Zn parents, such as inbred 1 and 2 from the high-Zn QPM group and 16 and 20 from the high-Zn non-QPM group. Similar observations were reported in maize [61] and pearl millet [62–65]. However, the Zn levels were lower for all hybrids derived from high-Zn lines compared to the respective values observed in their parental inbred lines. This is consistent with previous studies in maize [61,66] and pearl millet [63,65] which reported significantly lower Zn in hybrids compared to their parental inbred lines. Therefore, an additional criterion, evaluation in hybrid combinations, should be considered when selecting inbred lines for use as parents of hybrids with higher Zn content.

Nutritional improvement in crop plants, including Zn-enriched maize hybrids may result in a yield penalty [8]. However, a wide range of studies have reported that yield, and nutritional traits such as kernel Zn could be improved simultaneously [67–69]. In this study, grain yield was not correlated with Zn (r=0.02). Similar observations were reported in previous studies of maize [27,61,70–72] and pearl millet [64]. Lack of correlation between grain yield and Zn suggested the possibility of improving maize for Zn concentration without reducing the grain yield potential of the hybrids. Consistent with previous studies, hybrids with elevated Zn and grain yield have been reported [7,61,73].

Hybrids developed from mating high-Zn (QPM x non-QPM) inbreds had enhanced levels of Zn concentration. Increased levels of Zn have been reported for QPM germplasm compared to non-QPM germplasm [39–41,43]. The dominant, wild-type allele of the *o2* locus codes for a transcriptional factor that regulates the synthesis of zeins [74].

In genotypes homozygous recessive at the *o2* locus, there is a decrease in α-zein [75] with a proportional increase of non-zeins such as albumins, glutelins and globulins [76]. Those non-zeins are known to bind Zn in the endosperm [77]. Thus, in QPM inbreds, and possibly in some of their hybrids, the elevated levels of Zn could be attributed to reduced levels of zeins and relatively higher levels of other Zn-binding proteins [40].

Higher levels of Zn have also been reported in non-QPM inbred lines [44,48–50,78]. For such inbreds, and perhaps in their hybrids, higher Zn levels could be attributed to genetic factors unrelated to the *o2* locus. Therefore, it might be helpful to explore other mechanisms that can potentially account for high-Zn in those genotypes. During grain filling, metal-binding proteins such as metallothioneins, phytochelatins and nicotianamine are thought to bind Zn in large amounts [79]. The storage capacity of those binding proteins could possibly be associated with the amount of Zn that accumulates in a maize kernel. Genotypes with a high capacity for Zn storage may possess more Zn-binding proteins. Consequently, enhanced levels of Zn may be achieved in genotypes with more Zn-binding proteins than genotypes with less Zn-binding proteins [40]. Instead, enhanced levels of Zn in those hybrids may be attributed to the increase in Zn-binding capacity because of the metal-binding proteins.

Also, other possible sources of higher levels of Zn in kernels may be attributable to disproportionate growth of the endosperm and embryo. In maize, approximately 49% of the total kernel Zn is in the embryo and the remainder is in the endosperm [19]. If either tissue grows in an unexpected and disproportionate manner, the total amount of Zn in the kernel could be affected. Inbred lines and their hybrid progeny often display different phenology and durations of developmental stage.

For example, it is well-known that inbred lines flower later and are shorter than their hybrid progeny. So, in addition to the possibility of Zn-binding proteins, the relative proportions of embryo and endosperm in the hybrid progeny should be considered in future investigations.

Understanding the nature of gene action responsible for Zn accumulation in maize kernels could be important in designing an effective breeding strategy for hybrids with increased Zn. The GCA effects accounted for ≥ 70% of the total variability suggesting that the accumulation of Zn in maize kernels is predominantly governed by additive gene effects. Similar results were reported in maize [26–28,32,48,80], pearl millet [62–64], rice [81,82], sorghum [83] and wheat [84]. With predominance of variance due to GCA, hybrids with enhanced Zn levels can be obtained by crossing parents with positive GCA effects [85,86].

Among the 10 inbred lines that were originally classified as high-Zn parents, only three inbreds, namely, inbred 2 from the high Zn-QPM group and 16 and 20 from the high-Zn non-QPM group had positive GCA effects. This observation was contrary to an earlier study involving 14 inbred lines in which positive GCA effects were observed for all high-Zn parents (seven), while significantly negative GCA effects were detected for the low-Zn lines [28]. Also, positive GCA effects for Zn were detected for inbreds 8, a low-Zn QPM, and 11 and 13 both from the low-Zn non-QPM group. The positive GCA for Zn observed for inbred lines 2, 8,11,13,16 and 20 suggest possibility of transmitting favorable alleles from these parental lines to their hybrids and could be useful for breeding to improve Zn content.

Kernel Zn is a phenotype determined late in the development of a maize crop, subject to environmental influences, requiring extensive sample preparation, trained analysts and costly equipment. Therefore, it could be helpful to identify a secondary trait that can potentially be used for indirect selection of Zn.

During growth and development of a maize plant, the vegetative parts serves as a primary source of Zn for kernels. Consequently, plant height could conceivably be used as a secondary trait associated with Zn in kernels. Taller maize hybrids may have more Zn stored in their vegetative parts. Hence, more Zn may be remobilized to the kernels of taller plants than kernels of shorter plants. However, in this study, there was no correlation between plant height and Zn concentration as noted in previous research [71]

In summary, the inbreds' phenotype may provide some useful information for developing hybrids with increased Zn content, although more reliable results can be obtained by evaluating the inbreds in hybrid combinations. Hybrids derived from crossing QPM inbred lines alone had greater mean values for Zn (26.93 μg/g) than hybrids derived from crossing non-QPM inbred lines (22.70 μg/g). However, hybrids with the highest mean values for Zn were observed when high-Zn QPM inbred lines were crossed with high-Zn non-QPM inbreds (hybrids 51, 56 and 60 had ≥ 30 μg/g of Zn). Six inbred lines with positive and/or significant GCA for Zn were identified. Taken together, these results indicate some potential to develop high-Zn hybrids using a combination of QPM and non-QPM inbred lines. The largest proportion of variability for Zn

Authors Contributions

T.D., F.V., and M.L conceived and designed the experiments; T.D., and N.P., conducted all the field and lab experiments and generated the data; E.M., J. B., and A.R., analyzed the data; E.M., wrote the manuscript; M.L., N.P., F.V., T.D., edited and reviewed the manuscript.

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CHAPTER 3. GENOMIC PREDICTION WITH GENOTYPE BY ENVIRONMENT INTERACTION ANALYSIS FOR KERNEL ZINC CONCENTRATION IN TROPICAL MAIZE GERMPLASM

Manuscript under review Genes | Genomes | Genetics (G3) Journal

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Abstract

Zinc (Zn) deficiency is a major risk factor for human health, affecting about 30% of the world's population. To study the potential of genomic selection (GS) for maize with increased Zn concentration, an association panel and two doubled haploid (DH) populations were evaluated in three environments. Three genomic prediction models, M (M1: Environment +

Line, M2: Environment + Line + Genomic, and M3: Environment + Line + Genomic + Genomic x Environment) incorporating main effects (lines and genomic) and the interaction between genomic and environment (G x E) were assessed to estimate the prediction ability (r_{MP}) for each model. Two distinct cross-validation (CV) schemes simulating two genomic prediction breeding scenarios were used. CV1 predicts the performance of newly developed lines, whereas CV2 predicts the performance of lines tested in sparse multi-location trials. Average prediction accuracies were 0.71, 0.70 and 0.51 for the association mapping panel, DH1 and DH2, respectively. The genomic prediction model which included G x E interaction marginally increased the prediction accuracies for both CV1 (0.37 to 0.39 and 0.43 to 0.44) and CV2 (0.69 to 0.71 and 0.50 to 0.51) for the association panel and DH2 population, respectively. These results suggest that GS has potential to accelerate breeding for enhanced kernel Zn concentration by facilitating selection of superior genotypes.

Introduction

Malnutrition arising from zinc (Zn) deficiency is a major risk factor for human health affecting nearly 20% of the world's population (Bouis and Saltzman 2017; Gannon *et al.* 2017). The problem is more prevalent in low-and middle income countries (LMICs), and is highly attributed to lack of access to a balanced diet, reliance on cereal-based diets and ignorance of good nutritional practices (Welch and Graham 2004). Several approaches, such as food fortification, diversification and supplementation have been tried to reduce Zn deficiency. However, in LMICs, these methods have not been entirely successful (Misra *et al.* 2004; Stein 2010).

Breeding maize for increased Zn concentration may offer some relief. The Zn-enriched varieties can be widely accessible, will not require continued investment once developed, and

they remain after the initial successful investment and research (Govindan 2011). Recently, maize varieties with 15-36% more Zn were released in Guatemala and Colombia (Listman 2019). Nevertheless, increased breeding efforts are required to develop more Zn-enriched varieties for a diverse range of environments and management practices. Progress toward developing those varieties has mainly relied upon conventional plant breeding approach that is labor-intensive and time-consuming. However, with the recent advances in genomics, new methods for plant breeding such as genomic selection (GS) can be used to identify genotypes with enhanced Zn concentration more efficiently and rapidly.

In a GS breeding scheme, genome-wide DNA markers are used to predict which individuals in a breeding population are most valuable as parents of the next generation (cycle) of offspring (Meuwissen *et al.* 2001; de los Campos *et al.* 2009; Pérez-Rodríguez *et al.* 2012). Kernel Zn concentration is determined at the end of a plant's life cycle, so GS can enable selection of promising genotypes earlier in the life cycle. This reduces the time and cost of phenotypic evaluation and may increase the genetic gain per unit time and cost (Heslot *et al.* 2015; Manickavelu *et al.* 2017; Arojju *et al.* 2019).

The utility and effectiveness of GS has been examined for many different crop species, marker densities, traits and statistical models and varying levels of prediction accuracy have been achieved (de los Campos *et al.* 2009, 2013; Crossa *et al.* 2010, 2013, 2014; Jarquín *et al.* 2014; Pérez-Rodríguez *et al.* 2015; Zhang *et al.* 2015; Velu *et al.* 2016). Although the number of markers needed for accurate prediction of genotypic values depends on the extent of linkage disequilibrium between markers and QTL (Meuwissen *et al.* 2001), a higher marker density can improve the proportion of genetic variation explained by markers and thus result in higher prediction accuracy (Albrecht *et al.* 2011; Zhao *et al.* 2012; Combs and Bernardo 2013; Liu *et al.*

2018). Importantly, higher prediction accuracies have been obtained when genotypes of a population are closely related than when genetically unrelated (Pszczola *et al.* 2012; Combs and Bernardo 2013; Spindel and McCouch 2016).

Initially, GS models and methods were developed for single-environment analyses and they did not consider correlated environmental structures due to genotype by environment (G x E) interactions (Crossa *et al.* 2014). The differential response of genotypes in different environments is a major challenge for breeders and can affect heritability and genotype ranking over environments (Monteverde *et al.* 2018). Multi-environment analysis can model G x E using genetic and residual covariance functions (Burgueño *et al.* 2012), markers and environmental covariates (Jarquín *et al.* 2014), or marker by environment (M x E) interactions (Lopez-Cruz *et al.* 2015). This approach to GS can successfully be used for biofortification breeding of maize because multi-environment testing is routinely used in the development and release of varieties.

Modelling covariance matrices to account for G x E allows the use of information from correlated environments (Burgueño *et al.* 2012). Mixed models that allow the incorporation of a genetic covariance matrix calculated from marker data, rather than assuming independence among genotypes improves the estimation of genetic effects (VanRaden 2008). The benefit of using genetic covariance matrices in G x E mixed models is that the model relates genotypes across locations even when the lines are not present in all locations (Monteverde *et al.* 2018). GS models capable of accounting for multi-environment data have extensively been studied in different crops (Zhang *et al.* 2015; Cuevas *et al.* 2016, 2017; Velu *et al.* 2016; Jarquín *et al.* 2017; Sukumaran *et al.* 2017a; Monteverde *et al.* 2018; Roorkiwal *et al.* 2018). In those studies, incorporating G x E demonstrated a substantial increase in prediction accuracy relative to single-environment analyses.

Kernel Zn has been investigated in several quantitative trait loci (QTL) analyses in maize and each study has reported that Zn concentration is under the control of several loci. The phenotypic variation explained by those loci ranges from 5.9 to 48.8% (Zhou *et al.* 2010; Qin *et al.* 2012; Ŝimić *et al.* 2012; Baxter *et al.* 2013; Jin *et al.* 2013; Zhang *et al.* 2017a; Hindu *et al.* 2018). A Meta-QTL analysis across several of those studies identified regions on chromosome 2 that might be important for kernel Zn concentration (Jin *et al.* 2013). Additionally, genomic regions associated with Zn concentration were recently reported in a genome-wide association study of maize inbreds adapted to the tropics (Hindu *et al.* 2018). Whereas some of the regions were novel, four of the twenty identified were located in already reported QTL intervals. Taken together, the QTLs may be used in a breeding program through marker-assisted selection (MAS) or GS.

A wide array of maize genetic studies has reported considerable effects of G x E interactions for kernel Zn concentration (Oikeh *et al.* 2003, 2004; Long *et al.* 2004; Chakraborti *et al.* 2009; Prasanna *et al.* 2011; Agrawal *et al.* 2012; Guleria *et al.* 2013). However, genotypes with high-Zn concentration have been identified in both tropical and temperate germplasm (Ahmadi *et al.* 1993; Bänziger and Long 2000; Brkic *et al.* 2004; Menkir 2008; Chakraborti *et al.* 2011; Prasanna *et al.* 2011; Hindu *et al.* 2018). Additionally, evaluation procedures for kernel Zn are labor-intensive, expensive and time-consuming (Palacios-Rojas 2018). To the best of our knowledge, no study has examined the predictive ability of GS methods that incorporate G x E for Zn concentration in maize. Within the framework of the reaction norm model (Jarquín *et al.* 2014), the potential of GS for Zn using maize inbreds adapted to tropical environments were assessed. The objectives of this study were; (i) to evaluate the prediction ability for Zn using an association mapping panel and two bi-parental populations evaluated in three tropical

environments, (ii) to assess and compare the predictive ability of different GS models, and (iii) to examine the effects of incorporating G x E on prediction accuracy for Zn.

Materials and Methods

Zinc association mapping (ZAM) panel

The ZAM panel consists of 923 inbreds from maize breeding programs of the International Maize and Wheat Improvement Center (CIMMYT). The panel represents wide genetic diversity for kernel Zn concentration (Hindu *et al.* 2018).

Bi-parental DH populations

From the ZAM panel, four inbreds with contrasting Zn concentration were selected and used to form two bi-parental (doubled haploid [DH]) populations (Table 3. 1). DH1 was derived from the F1 generation of a mating between CML503, a high-Zn inbred (31.21 μg/g) with CLWN201, a low-Zn inbred (22.62 μg/g). DH2 was derived from the F1 generation of a mating between CML465, another high-Zn inbred (31.55 μg/g) with CML451, a moderate-Zn inbred (27.88 μg/g). DH1 and DH2 were comprised of 112 and 143 inbreds, respectively.

Table 3.1 Pedigree and average concentration of kernel Zn (μg/g) concentration for the parents of the DH populations

DH population	Pedigree	Parent1	Parent2	Zn (μg/g)	
				Parent1	Parent2
DH1	CML503/CLWN201	CML503	CLWN201	31.21	22.62
DH2	CML 465/CML451	CML465	CML451	31.55	27.88

Experimental design and phenotypic evaluation

Zinc association mapping (ZAM) panel

The ZAM panel was grown at CIMMYT research stations in Mexico, during the months of June through September and November through March at Agua Fria in 2012 and 2013, and Celaya in 2012. Plot sizes and the experimental designs (Hindu *et al.* 2018).

Bi-parental DH populations

The DH populations were grown at CIMMYT research stations in Mexico; Celaya in 2014 and Tlaltizapan (18°41'N, 99° 07′ W; 962.5 m asl) in 2015 and 2017. In 2014 and 2015, both populations were evaluated in single-replication trials (Hindu *et al.* 2018). In 2017, a randomized complete block design (RCBD) with two replications was used. The rows were 2.5 m long and 75 cm apart and each genotype was grown in a single row plot. All plots were managed according to the recommended agronomic practices for each environment.

From the ZAM panel and each DH population, four to six plants in each plot were self-pollinated, hand-harvested at physiological maturity, hand-shelled and dried to a moisture content of 12.5%. The bulked kernels from each plot are considered a representative sample and were used in subsequent Zn analyses as described (Hindu et al. 2018).

Genotypic data

Genomic DNA was extracted from leaf tissues of all inbred lines (ZAM panel and DH populations) using the standard CIMMYT laboratory protocol (CIMMYT, 2005). The samples were genotyped using the genotyping by sequencing (GBS) method at the Institute for Genomic Diversity, Cornell University, USA (Elshire *et al.* 2011; Crossa *et al.* 2013).

The restriction enzyme ApeK1 was used to digest DNA, GBS libraries were constructed

in 96-plex and sequenced on a single lane of Illumina HISeq2000 flow cell (Elshire *et al.* 2011). To increase the genome coverage and read depth for SNP discovery, raw read data from the sequencing samples were analyzed together with an additional ~30, 000 global maize collections (Zhang *et al.* 2015).

SNP identification was performed using TASSEL 5.0 GBS Discovery Pipeline with B73 (RefGen_v2) as the reference genome (Elshire *et al.* 2011; Glaubitz *et al.* 2014). The source code and the TASSEL GBS discovery pipeline are available at https://www.maizegenetics.net and the SourceForge Tassel project https://sourceforge.net/projects/tassel. For each inbred, the pipeline yielded 955, 690 SNPs which were distributed on the 10 maize chromosomes. After filtering using a minor allele frequency of 0.05 and removing SNPs with more than 10% missing data, 181,889 (ZAM panel) and 170, 798 (bi-parental) SNPs were used for genomic prediction.

Phenotypic data analysis

For the ZAM panel, broad-sense heritability *(H^2)* across environments was estimated as:

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + {\sigma_{GE}^2}/{l} + {\sigma_e^2}/{lr}}$$

where σ_G^2 is the variance due to genotype, σ_{GE}^2 is variance due to genotype x environment, σ_e^2 is the error variance, *l* is the number of environments and *r* is the number of replications using multi-environment trial analysis with R (META-R) (Alvarado *et al.* 2016). For the DH populations, variance components based on the genomic relationship matrix were computed using BGLR package as implemented in GBLUP (Pérez and de los Campos 2014).

An estimate of narrow-sense heritability ($\hat{h}^2$) for each DH population was calculated as:

$$\hat{h}^2 = \frac{\hat{\sigma}_g^2}{\hat{\sigma}_g^2 + \hat{\sigma}_e^2}$$

where $\hat{\sigma}_g^2$ is an estimate of the additive genetic variance and $\hat{\sigma}_e^2$ is an estimate of the residual variance.

Correlation coefficients between Zn and environments, descriptive statistics and phenotypic data distribution using boxplots were generated in R (core Team 2018). Line means (genotypic values) for the ZAM panel were estimated as Best Linear Unbiased Estimators (BLUEs) with a random effect for replications nested within each environment. Raw data (values) were used for the DH populations.

Statistical models

Genomic models used in this study were based on the reaction norm model which models the markers (genomic) by environment interaction (Jarquín *et al.* 2014). This model is an extension of the Genomic Best Linear Unbiased Predictor (GBLUP) random effect model, where the main effects of lines (genotypes), genomic, environments and their interactions are modelled using covariance structures that are functions of marker genotypes and environmental covariates. In this study, environment is the combination of site and year (site-by-year).
A brief description of the models is given below.

M0. Phenotypic baseline model

The phenotypes y_{ij} are modelled as:

$$y_{ij} = \mu + E_i + L_j + EL_{ij} + e_{ij}.$$

This linear model represents the response of the j^{th} (j=1,…,J) genotype/line tested in the i^{th} (i=1,…,I) environment and (y_{ij}) as the sum of an overall mean μ plus random environmental main effect $\left[E_i \overset{iid}{\sim} N(0, \sigma_E^2)\right]$, the random genotype main effect $\left[L_j \overset{iid}{\sim} N(0, \sigma_L^2)\right]$, the random interaction between the j^{th} genotype and the i^{th} environment $\left[EL_{ij} \overset{iid}{\sim} N(0, \sigma_{EL}^2)\right]$ and a random error term $\left[e_{ij} \overset{iid}{\sim} N(0, \sigma_e^2)\right]$. From this linear model, $N(.,.)$ denotes a normal random variable, iid stands for independent and identically distributed responses and σ_E^2, σ_L^2, σ_{EL}^2, σ_e^2 are the variances for environment, genotype, genotype by environment and residual error, respectively.

The baseline model does not allow borrowing of information among genotypes because the genotypes were treated as independent outcomes. Thus, models used in this study were derived from the baseline model by subtracting terms or modifying assumptions and/or incorporating genomics/marker information.

M1. Environment + Line

This model is obtained by retaining the first three components from the baseline model (overall mean, random environment main effect and random line main effect) while their underlying assumptions remain unchanged.

$$y_{ij} = \mu + E_i + L_j + e_{ij}. \qquad [1]$$

Here environments were considered as site-by-year combinations.

M2. Environment + Line + Genomic

Another representation of the random main effect of line L_j in the previous model is considering a linear combination between markers and their correspondent marker effects, $g_j = \sum_{m=1}^{p} x_{jm} b_m$, such that

$$y_{ij} = \mu + E_i + L_j + g_j + e_{ij} \qquad [2]$$

where $b_m \overset{iid}{\sim} N(0, \sigma_b^2)$ represents the random effect of the m^{th} (m=1,…,p) marker, x_{jm} is the genotype of the jth line at the mth marker and σ_b^2 its correspondent variance component. Therefore, $\mathbf{g} = (g_1, \ldots, g_J)'$, is the vector of genetic effects, and follows a normal density with mean zero, and a co-variance matrix $Cov(\mathbf{g}) = \mathbf{G}\sigma_g^2$ with $\mathbf{G} = \frac{\mathbf{XX}'}{p}$ being the genomic relationship matrix (Lopez-Cruz *et al.* 2015) that describes genetic similarities among pairs of individuals. In this model, the line effect L_j is retained to account for imperfect information and model mis-specification because of potential imperfect linkage disequilibrium between markers and quantitative trait loci (QTLs).

M3. Environment + Line + Genomic + Genomic × Environment

This model accounts for the effects of lines L_j, of markers (genomic) g_j, of environments (E_i) and the interaction between markers (genomic) and the environment (Eg_{ij}). The model includes the interaction between markers (genomics) and the environment via co-variance structure (Jarquín *et al.* 2014). The model is as follows:

$$y_{ij} = \mu + E_i + L_j + g_j + Eg_{ij} + e_{ij} \qquad [3]$$

Where Eg_{ij} is the interaction between the genetic value of the i^{th} genotype in the j^{th} environment and $\boldsymbol{Eg} = \{Eg_{ij}\} \sim N\left(\mathbf{0}, \left(\boldsymbol{Z_g G Z_g'}\right) \# \left(\boldsymbol{Z_E Z_E'}\right)\sigma_{Eg}^2\right)$, where $\boldsymbol{Z}_g$ and $\boldsymbol{Z}_E$ are the correspondent incidence matrices for the effects of genetic values of genotypes and environments, respectively, σ_{Eg}^2 is the variance component of $\boldsymbol{Eg}$ and # denotes the Hadamard product (element-to-element product) between two matrices.

Prediction accuracy assessment using cross-validation

Two distinct cross-validation schemes that mimic prediction problems that breeders may face when performing genomic prediction were used (Burgueño *et al.* 2012). One random cross-validation (CV1) evaluates the prediction ability of models when a set of lines have not been evaluated in any environment (prediction of newly developed lines). In CV1, predictions are entirely based on phenotypic records of genetically related lines. The second cross-validation (CV2) is related to incomplete field trials also known as sparse testing, in which some lines are observed in some environments but not in others. In CV2, the goal is to predict the performance of lines in environments where they have not yet been observed. Thus, information from related lines and the correlated environments is used, and prediction assessment can benefit from borrowing information between lines within an environment, between lines across environments and among correlated environments.

In CV1 and CV2, a fivefold cross-validation scheme was used to generate the training and validation sets to assess the prediction ability for Zn within the ZAM panel and each DH population. The data were randomly divided into five subsets, with 80% of the lines assigned to the training set and 20% assigned to the validation set. Four subsets were combined to form the training set, and the remaining subset was used as the validation set. Permutation of five subsets taken one at a time led to five training and validation data sets. The procedure was repeated 20 times and a total of 100 runs were performed in each population. The average value of the correlations between the phenotype and the genomic estimated breeding values (GEBVs) from 100 runs was calculated for the ZAM panel, and each DH population for Zn in each environment and was defined as the prediction ability (r_{MP}).

Data availability

All models were fitted in R (core Team 2018) using the BGLR package (Pérez and de los Campos 2014). All phenotypic and genomic data can be downloaded from the link: http://hdl.handle.net/11529/10548331

Results

Descriptive statistics

Mean values of kernel Zn concentration were estimated for each environment and across environments (Tables 3.2A and 3.2B). For the ZAM panel, kernel Zn ranged from 14.76 to 39.80 µg/g in Celaya 2012, 15.16 to 42.52 µg/g and 17.05 to 46.52 µg/g in Agua Fria 2012 and 2013, respectively (Figure 3.1). The highest mean (29.53 µg/g) for Zn was observed in Agua Fria 2013.

DH1 had Zn values ranging from 16.00 to 48.00 µg/g in Celaya 2012, 16.00 to 35.00 µg/g in Tlaltizapan 2015 and 15.50 to 39.00 µg/g in Tlaltizapan 2017, while the respective values for DH 2 were 17.70 to 43.14 µg/g, 15.60 to 37.80 µg/g and 14.70 to 37.60 µg/g (Figures 3.2A and 3.2B). The highest means for Zn were observed in Celaya 2014 (25.38 µg/g) and 2017 (27.96 µg/g) for DH1 and DH2, respectively, (Table 3.2B). Across environments, heritability ($H^2/\widehat{h^2}$) estimates were 0.85, 0.83 and 0.76 for the ZAM panel, DH1 and DH2, respectively (Tables 3.2A and 3.2B). There were significant positive correlations between environments for Zn (Table 3. 3), accounting for the moderate to high heritability estimates.

Table 3.2 Descriptive statistics for kernel Zn concentration in (A) the ZAM panel and (B) DH populations grown in each environment, variance components and broad-and narrow sense heritabilities

A

Population	Population size	Location	Mean ± se (µg/g)	σ_G^{2a}	σ_{GE}^{2a}	H^2
ZAM panel	923	Agua Fria 2012	26.15 ± 0.15	12.04	2.42	0.85
		Celaya 2012	25.06 ± 0.14			
		Agua Fria 2013	29.53 ± 0.16			
		Across	**26.94 ± 0.10**			

B

Population	Population size	Location	Mean ± se (µg/g)	$\hat{h}^2$
DH1	112	Celaya 2014	25.38 ± 0.48	0.83
		Tlaltizapan 2015	24.01 ± 0.38	
		Tlaltizapan 2017	24.53 ± 0.37	
		Across	**24.65 ± 0.26**	
DH2	143	Celaya 2014	27.96 ± 0.39	0.76
		Tlaltizapan 2015	24.08 ± 0.33	
		Tlaltizapan 2017	24.64 ± 0.37	
		Across	**25.59 ± 0.22**	

Broad-sense heritability H^2 of Zn in each environment and across environments
Narrow-sense heritability $\widehat{h^2}$ of Zn across environments
[a]variance due to genotypes σ_G^2 and the interaction between genotypes and the environment σ_{GE}^2 significant at P<0.001

Table 3.3 Phenotypic correlation between environments for kernel Zn

Environment	DH1	DH 2	ZAM Panel
[a]Env1 vs Env2	0.62	0.46	0.63
[a]Env1 vs Env3	0.58	0.29	0.66
[a]Env2 vs Env3	0.62	0.45	0.61

Phenotypic correlation coefficients were significant at α = 0.001
[a]DH populations; Env 1, Env2 and Env 3=Celaya,2014, Tlaltizapan, 2017 and Tlaltizapan 2017, respectively.
[a]ZAM panel; Env 1, Env2 and Env 3= Agua Fria, 2012, Celaya, 2012 and Agua Fria 2013, respectively.

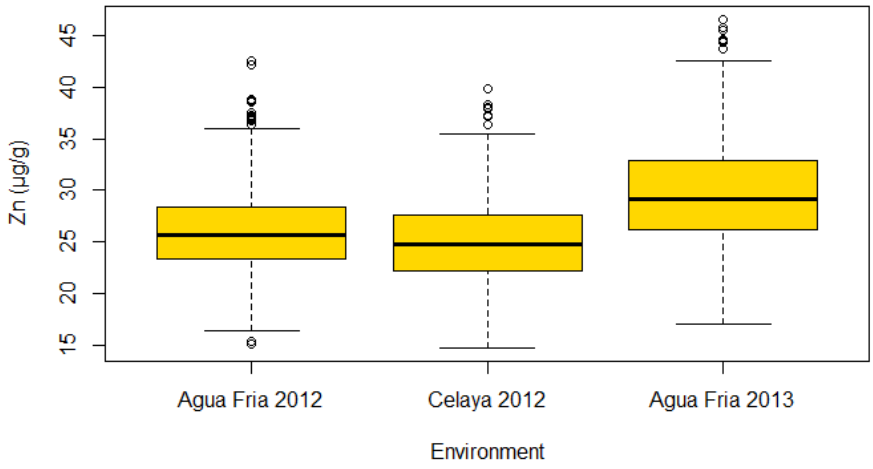

Figure 3.1 Box plots for kernel Zn (µg/g) in the ZAM panel in three environments (Agua Fria, 2012, Celaya, 2012 and Agua Fria, 2013)

A

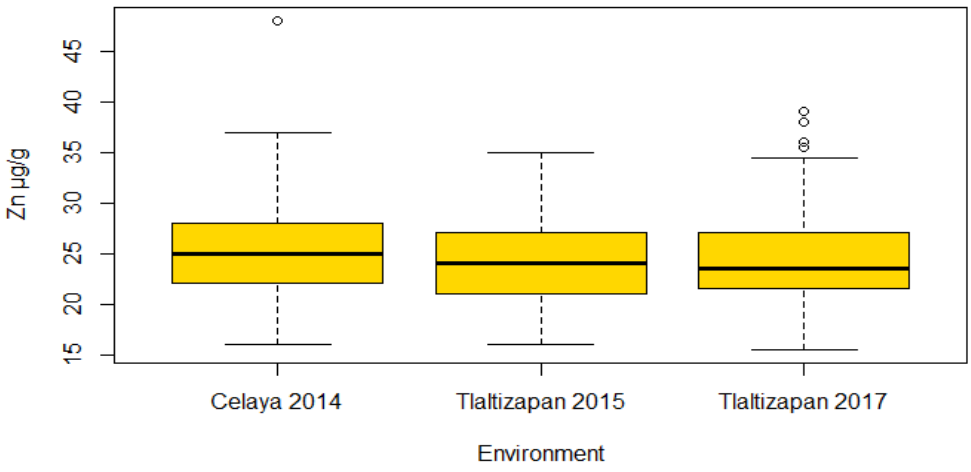

Figure 3.2 (A) Box plots for kernel Zn (µg/g) for DH1 in three environments (Celaya 2014, Tlaltizapan, 2015 and Tlaltizapan, 2017)

B

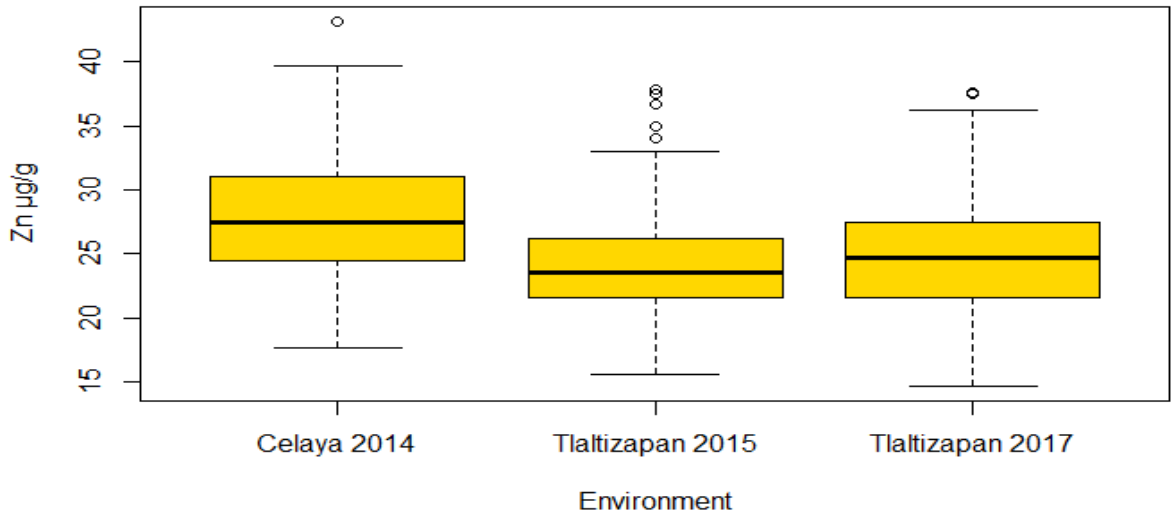

Figure 3.2 (B) Box plots for kernel Zn (µg/g) for DH2 in three environments (Celaya 2014, Tlaltizapan, 2015 and Tlaltizapan, 2017)

Principal component analysis for the ZAM panel suggested presence of a relatively diverse set of lines, and 452 principal components (PCs) were needed to explain 80% of the genotypes' variance (Figures 3.3A and 3.3B). The first two principal components explained 3.85% of the total variance. For the DH populations first two eigenvectors separated them two groups (DH1 and DH2) and 56 principal components were needed to explain 80% of the genotypes' variance (Figures 3.3C and 3.3D). The first two principal components explained 27.50% of the total variation for the DH populations.

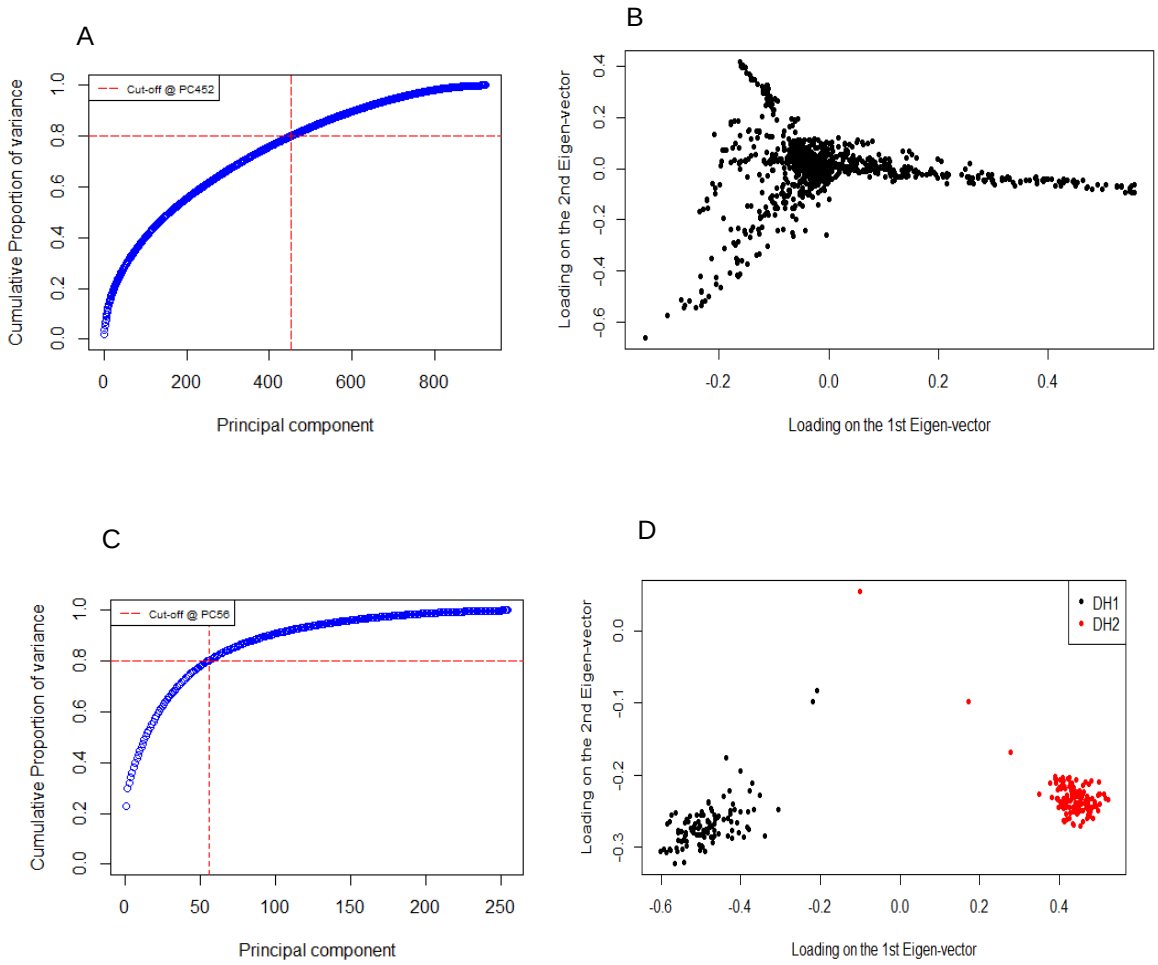

Figure 3.3 Scree plots (A and C) and loadings of the first two eigenvectors (B and D) of the covariance matrices derived from markers for the ZAM panel (A and B) and for the DH populations (C and D)

Prediction ability in different populations

Cross-validated r_{MP} values for kernel Zn were estimated for the ZAM panel and DH populations (Tables 3.4, 3.5 and 3.6). The average r_{MP} values in CV1 were consistently lower than those in CV2, suggesting the importance of using information from correlated environments when predicting performance of inbred lines. The mean r_{MP} values in CV1 and CV2 for the

ZAM panel were 0.39 and 0.71, respectively (Table 3.4). For the DH populations, average r_{MP} values were 0.53 for DH1-CV1, 0.44 for DH2-CV1 (Table 3.5), 0.70 for DH1-CV2 and 0.51 for DH2-CV2 (Table 3.6). In the ZAM panel, the highest values in CV1 (0.47) and CV2 (0.72) were obtained in Celaya and Agua Fria 2012 (Table 3.4).

Table 3.4 Correlations (mean ± SD) between observed and genomic estimated breeding values for kernel Zn in the three environments for three GBLUP models for cross-validations CV1 and CV2 of the ZAM panel

		Prediction accuracy in CV1		
Population	Environment	M1	M2	M3
ZAM panel (923)	Agua Fria, 2012	-0.01± 0.04	0.33 ± 0.01	0.34 ± 0.02
	Celaya, 2012	0.004 ± 0.04	0.43 ± 0.01	0.47 ± 0.01
	Agua Fria, 2013	-0.001 ± 0.03	0.34 ± 0.01	0.35 ± 0.01
	Average	**-0.001± 0.03**	**0.37 ± 0.01**	**0.39 ± 0.01**
		Prediction accuracy in CV2		
Population	Environment	M1	M2	M3
ZAM panel (923)	Agua Fria, 2012	0.71 ± 0.00	0.71 ± 0.00	0.72 ± 0.00
	Celaya, 2012	0.64 ± 0.00	0.68 ± 0.00	0.72 ± 0.00
	Agua Fria, 2013	0.67 ± 0.00	0.67 ± 0.00	0.69 ± 0.01
	Average	**0.67 ± 0.00**	**0.69 ± 0.00**	**0.71 ± 0.00**

Models: M1= Environment +Line; M2 = Environment + Line + Genomic; M3 = Environment + Line + Genomic + Genomic × Environment

For the bi-parental populations, both under CV1 and CV2, higher r_{MP} values were observed for DH1 compared to DH2. The highest values in CV1 (0.56) and CV2 (0.71) were observed in Tlaltizapan 2017 and 2015, all for DH1 (Tables 3.5 and 3.6). The consistently higher r_{MP} values in CV1 and CV2 of DH1 could be attributed to the higher (0.58 to 0.62) correlation values between environments (Table 3.3).

Table 3.5 Correlations (mean ± SD) between observed and genomic estimated breeding values for Zn in the three environments for three GBLUP models for cross-validation CV1 of DH populations

Population	Environment	Prediction accuracy in CV1		
		M1	M2	M3
DH1	Celaya, 2014	-0.05 ± 0.10	0.52 ± 0.04	0.51 ± 0.04
	Tlaltizapan, 2015	-0.02 ± 0.12	0.52 ± 0.05	0.51 ± 0.05
	Tlaltizapan, 2017	-0.01 ± 0.10	0.56 ± 0.05	0.55 ± 0.05
	Average	**-0.03 ± 0.10**	**0.53 ± 0.04**	**0.52 ± 0.04**
DH2	Celaya, 2014	0.05 ± 0.08	0.47 ± 0.03	0.50 ± 0.04
	Tlaltizapan, 2015	0.03 ± 0.08	0.45 ± 0.03	0.45 ± 0.03
	Tlaltizapan,2017	0.04 ± 0.08	0.35 ± 0.03	0.35 ± 0.04
	Average	**0.04 ± 0.06**	**0.43 ± 0.03**	**0.44 ± 0.02**

Models: M1= Environment +Line; M2 = Environment + Line + Genomic; M3 = Environment + Line + Genomic + Genomic × Environment

Prediction ability of different models

Comparing the r_{MP} values obtained from each model, M1 had the lowest (-0.001, -0.03 and 0.04) accuracies in CV1 for the ZAM panel and DH populations (Tables 3.4 and 3.5). Those values were improved in CV2 because the predictions benefited from previous records (collected from other environments) of lines whose Zn values were being predicted. When M1 was expanded to M2 by adding the main effects of markers, the r_{MP} values at each environment and across environments were increased. For example, in CV1, M2, >100-fold increase in r_{MP} values were observed for the ZAM panel and DH populations, and in CV2, M2, average r_{MP} values increased by 2.98%, 2.94% and 11.11% for the ZAM panel, DH1 and DH2, respectively (Tables 3.4, 3.5 and 3.6).

Table 3.6 Correlations (mean ± SD) between observed and genomic estimated breeding values for Zn in the three environments for three GBLUP models for cross-validation CV2 of DH populations

Population	Environment	Prediction accuracy in CV2		
		M1	M2	M3
DH1	Celaya, 2014	0.67 ± 0.02	0.68 ± 0.02	0.68 ± 0.03
	Tlaltizapan, 2015	0.70 ± 0.02	0.71 ± 0.02	0.70 ± 0.02
	Tlaltizapan, 2017	0.67 ± 0.02	0.70 ± 0.02	0.69 ± 0.02
	Average	**0.68 ± 0.01**	**0.70 ± 0.01**	**0.69 ± 0.01**
DH2	Celaya, 2014	0.46 ± 0.016	0.53 ± 0.02	0.56 ± 0.02
	Tlaltizapan, 2015	0.50 ± 0.020	0.55 ± 0.02	0.55± 0.02
	Tlaltizapan, 2017	0.40 ± 0.023	0.43 ± 0.02	0.43 ± 0.02
	Average	**0.45 ± 0.02**	**0.50 ± 0.01**	**0.51 ± 0.01**

Models: M1= Environment +Line; M2 = Environment + Line + Genomic; M3 = Environment + Line + Genomic + Genomic × Environment

The multi-environment model (M3), which includes the interaction between markers (genomic) and the environment (Eg_{ij}) marginally gave a higher prediction accuracy than the single-environment models (M1 and M2). In CV1, mean r_{MP} values slightly increased from 0.37 (M2) to 0.39 (M3) for the ZAM panel and from 0.43 (M2) to 0.44 for DH2 (Tables 3. 4 and 3. 5). Similar trends were observed in CV2 for the ZAM panel and DH2 (Tables 3. 4 and 3. 6). However, in both CV1 and CV2 of DH1, incorporating Eg_{ij} did not increase r_{MP} values for Zn (Tables 3. 5 and 3. 6). For CV1, M3, r_{MP} values for Zn in individual environments ranged from 0.34 to 0.47 for the ZAM panel (Table 3. 4), 0.51 to 0.55 for DH1 and 0.35 to 0.50 for DH2 (Table 3. 5). For CV2, M3, those values ranged from 0.69 to 0.72 for the ZAM panel, 0.68 to 0.70 for DH1 and 0.43 to 0.56 for DH2 (Tables 3. 4, 3. 5 and 3. 6).

Discussion

Overall, moderate to high prediction ability values for kernel Zn were observed for the ZAM panel and DH populations. This could be attributed to the heritabilities observed for kernel Zn (Tables 2A and 2B). Similar observations were reported for Zn concentration in wheat (Velu *et al.* 2016; Manickavelu *et al.* 2017). High quality predictions with high accuracy for GS programs are expected for traits with moderate to higher heritability estimates (Combs and Bernardo 2013; Lian *et al.* 2014; Muranty *et al.* 2015; Saint Pierre *et al.* 2016; Manickavelu *et al.* 2017; Zhang *et al.* 2017b, 2019; Arojju *et al.* 2019). Consistent with a study on Zn and iron (Fe) concentration in spring wheat, the prediction accuracies in this study are sufficient to discard at least 50% of the inbreds with low-Zn concentration (Velu *et al.* 2016).

Data from both bi-parental populations and diverse collection of inbreds have been used for GS and cross-validation (CV) experiments have shown that prediction accuracies could also be affected by the relatedness between training and prediction sets (Habier *et al.* 2007; de Roos *et al.* 2009; Asoro *et al.* 2011; Daetwyler *et al.* 2013; Cericola *et al.* 2017; Crossa *et al.* 2017). In this study, average predicted accuracies were higher for CV1 of the bi-parental populations (0.53 for DH1 and 0.44 for DH2) compared to the ZAM panel (0.39). Higher predicted values in CV1 of the DH populations could be attributed to the closer relationship between DH lines in the training and prediction sets, maximum linkage disequilibrium (LD) between a marker and a QTL, and controlled population structure (Bernardo and Yu 2007; Albrecht *et al.* 2011; Zhang *et al.* 2015). In collections of diverse inbreds, prediction accuracy may depend on the ancestral relationships between the lines. So, in experiments using such collections of lines, prediction accuracies have been more variable than accuracies achieved using bi-parental populations (Spindel and McCouch 2016).

Cross-validation (CV) schemes are used in genomic prediction to estimate the accuracy with which predictions for different traits and environments can be made (Burgueño *et al.* 2012; Zhang *et al.* 2015; Saint Pierre *et al.* 2016; Velu *et al.* 2016; Sukumaran *et al.* 2017a, 2017b; Monteverde *et al.* 2018; Roorkiwal *et al.* 2018). In this study, two CV schemes (CV1- predicting the performance of newly developed lines, and CV2- predicting the performance of lines that have been evaluated in some environments, but not in others) were used. The utility of these schemes indicated that prediction values for newly developed lines (CV1) were generally lower (0.39 for the ZAM panel, 0.53 for DH1 and 0.44 for DH2) than the values for lines which have been evaluated in different but correlated environments (CV2; 0.71, 0.70 and 0.51 for the ZAM panel, DH1 and DH2, respectively). Such observations indicate the importance of using information from correlated environments when predicting the performance of inbred lines. However, selection of new lines without field testing, as simulated in CV1 allows shortening of the generation interval (cycle time) by replacing the time-intensive phenotypic evaluation for Zn with genomic-estimated breeding values. But, the quality of prediction accuracy may be lower such that the annual rate of genetic progress in a GS program is compromised (Burgueño *et al.* 2012). So, the ultimate decision of how a breeding scheme should be structured could depend on the compromise between the desired prediction accuracy and the generation interval (Burgueño *et al.* 2012).

Genotype by environment interaction is an important factor affecting kernel Zn concentration in maize and genomic prediction models that incorporate G x E may enhance the potential of GS for biofortification breeding. For different crop species and traits, genomic prediction models which incorporated G x E achieved higher prediction accuracies in both CV1 and CV2 schemes relative to models which did not include G x E (Burgueño *et al.* 2012; Guo *et*

al. 2013; Jarquín *et al.* 2014; Lopez-Cruz *et al.* 2015; Zhang *et al.* 2015; Monteverde *et al.* 2018). In this study, the impact of modeling G x E variance structures for multi-environment trials was investigated and results indicated that the average predicted values from M3 (G x E model) were slightly higher (0.39 and 0.44 for CV1 and 0.71 and 0.51 for CV2) than the values from M2 (non-G x E; 0.37 and 0.43 for CV1-M2, 0.69 and 0.50 for CV2-M2) for the ZAM panel and DH2. These findings agree with those reported on Zn concentration in wheat (Velu *et al.* 2016), providing evidence that incorporating G x E in GS models can enhance their power and suitability for improving maize for kernel Zn concentration. Conversely, the average predicted values for CV1 and CV2 of DH1 were higher in M2 (0.53 and 0.70) than in M3 (0.53 and 0.69). Except for differences in population size (112 lines vs 143 lines), this was unexpected since DH1 and DH2 were grown in the same environments.

The gains in prediction accuracies for the GS model that accounted for G x E were dependent on the correlation between environments and CV method used. In this study, the phenotypic correlations between environments were all positive (ranging from 0.58 to 0.62 for DH1, 0.29 to 0.46 for DH2 and 0.61 to 0.66 for the ZAM panel). Such correlations can be exploited using multi-environment models to derive predictions that use information from across both the lines and environments (Burgueño *et al.* 2012). For instance, although the phenotypic correlations between environments for DH2 were positive (0.29 to 0.46), the lowest average prediction value (0.51) for CV2 was observed for this population. This was expected because CV2 uses phenotypic information from genotypes which have already been tested; hence, effectively exploiting the correlations between environments (Burgueño *et al.* 2012; Jarquín *et al.* 2014; Crossa *et al.* 2015; Pérez-Rodríguez *et al.* 2015; Saint Pierre *et al.* 2016; Monteverde *et al.* 2018). However, for CV1, the information between environments could only be accounted for

through the genomic relationship matrix (Monteverde *et al.* 2018). Hence, the gains in CV1 may likely attribute to more accurate estimate of environment-specific marker effects (Guo *et al.* 2013). In contrast, when multiple environments are weakly correlated, prediction accuracies from across environment analyses can be negatively affected relative to prediction accuracies within environments (Bentley *et al.* 2014; Wang *et al.* 2014; Spindel and McCouch 2016). Thus, before designing a GS experiment, identifying correlated environments where environments can differ in terms of site, year or season in which data were collected is of great interest (Spindel and McCouch 2016).

The ability to predict kernel Zn concentration using high-throughput SNP markers including G x E interactions creates an opportunity for efficiently enhancing Zn concentration in maize breeding programs. For instance, during early generations of a breeding program, GS can be utilized to identify genotypes with favorable alleles when numbers of progenies and families are large. This could potentially reduce the resource-intensive evaluation process and advancement of false-positive progenies (Velu *et al.* 2016). Coupled with advances in technologies for assessing Zn, plant scientists can more rapidly measure Zn concentration in maize kernels using the energy dispersive x-ray fluorescence (XRF) assays (Guild *et al.* 2017). Thus, with more validations and model refinements, GS can potentially accelerate the breeding process to enhance Zn concentration in maize for a wider range of environments.

Conclusion

The moderate to high prediction accuracies reported in this study shows that GS can be used in maize breeding to improve kernel Zn concentration. Assuming two possible seasons of Zn evaluation per year, the predicted genetic gains can be estimated from prediction accuracies

and genetic variances of the training populations. The genetic variances for the ZAM panel, DH1 and DH2 were 12.38, 12.20 and 14.88, and prediction accuracies were 0.71, 0.70 and 0.51, respectively. If the inbreds in each predicted population are ranked based on their predicted Zn values and the top 10% selected, then their expected average Zn values can be estimated from the proportion of inbreds selected, their respective training population genetic variances, prediction accuracies and the time interval for evaluating the lines. With reference to this, the expected average values of Zn are approximately 31 μg/g for the ZAM panel, 30 μg/g for DH1 and 27 μg/g for DH2. These averages are higher than the averages of the respective training populations (~27 μg/g for the ZAM panel, ~25 μg/g for DH1 and ~26 μg/g for DH2) suggesting that the prediction accuracies achieved are sufficient to select at least 10% of the predicted inbreds with higher Zn concentration.

The prediction accuracies were of lower quality when genomic predictions were conducted across populations. When the ZAM panel was used as the training population, prediction accuracies for DH1, DH2 and DH1+DH2 were 0.15, -0.10 and 0.09, respectively. When DH1 and DH2 were used as a training and prediction set for each other, prediction accuracies were 0.08 and 0.16 (Unpublished data). These prediction accuracies are considerably lower than those reported in this study and the differences may be attributed to: (i) weak genetic relationships between the training and prediction population sets and (ii) different methods of analysis because the prediction accuracies reported in this study were partly achieved by modelling the random-effects environment structure to account for G x E while for the unpublished data, the random-effects environment structure of G x E was not included.

This study also showed that higher prediction accuracies can be achieved when some of the lines are predicted using previous information about them collected from correlated

environments. The prediction model (M3) which included the interaction between markers and the environment marginally increased prediction accuracies in CV1 and CV2 for the association mapping panel and DH2 compared with the models which only included main effects (M1 and M2) indicating the importance of accounting for G x E in genomic prediction.

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CHAPTER 4. GENERAL CONCLUSIONS

A mating design study was conducted to assess the genetic basis of kernel Zn accumulation in maize. One hundred and forty-eight hybrids generated from mating QPM and non-QPM inbreds (high-Zn and low-Zn) were evaluated for kernel Zn concentration, grain yield and other traits. Results indicated that additive gene effects predominantly influenced kernel Zn accumulation. Additionally, there was no correlation between Zn and other traits such as grain yield, plant height and flowering time. The lack of correlation between Zn and these traits suggests the possibility of improving maize for Zn concentration without reducing the hybrid's potential for those traits. Direct evaluation of the inbreds' phenotype may provide useful information for breeding hybrids with elevated kernel Zn. However, more reliable results are obtained by evaluating the inbreds in hybrid combinations. Hybrids with improved kernel Zn can be developed using a combination of QPM and non-QPM inbred lines.

Using an association mapping panel and two-bi-parental doubled haploid (DH) populations, a genomic-enabled prediction study was conducted to assess the prediction ability for Zn. In maize, kernel Zn is determined at the end of a plant's life cycle, evaluation procedures are labor-intensive, expensive and time-consuming. So, genomic prediction can enable selection of promising genotypes earlier in the life cycle. Cross-validation schemes (CV1 and CV1) were used to estimate prediction accuracies for Zn. CV1 predicts the performance of lines that have not been evaluated in any environment, while CV2 predicts the performance of lines that have been evaluated in some environments, but not in others. Prediction models (M1-M3) incorporating main effects (lines and genomic/markers) and the interaction between genomic and environment (G x E) were assessed to estimate the prediction ability for Zn. The prediction abilities estimated through cross-validation were moderate to high suggesting the potential of

genomic prediction in Zn-biofortification breeding for maize. The multi-environment model (M3) which included the interaction between genomics/markers and the environment slightly gave a higher prediction accuracy both in CV1 and CV2 for the association panel and DH2 compared with the models which only included main effects (M1 and M2) indicating the importance of accounting for G x E in genomic prediction.

APPENDIX: SUPPLEMENTARY TABLES

Table A.1 Average kernel Zn concentration and grain yield for the hybrids in each environment

Hybrid	Cross	Zn (μg/g)					GY (t ha-1)				
		Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average	Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average
1	1 x 6	26.85	30.10	31.34	24.22	28.72	6.20	6.34	2.88	2.31	4.11
2	1 x 7	27.15	26.77	27.42	23.08	26.24	6.59	5.53	2.45	3.12	4.19
3	1 x 8	26.10	29.13	26.80	26.33	27.35	8.09	5.96	4.27	2.58	5.07
4	1 x 9	24.18	27.86	23.36	27.00	25.79	6.00	5.95	3.01	3.55	4.48
5	1 x 10	24.00	32.05	26.47	27.18	27.59	10.51	9.31	6.86	4.63	7.88
6	2 x 6	22.31	28.61	29.62	25.17	26.47	4.52	4.77	2.88	2.53	3.32
7	2 x 7	26.40	28.62	25.93	27.78	27.71	5.12	5.8	4.12	3.34	4.38
8	2 x 8	22.04	27.15	29.04	28.43	26.83	5.99	6.16	4.94	3.55	4.96
9	2 x 9	23.96	29.72	27.04	24.53	26.55	10.88	9.92	7.18	5.4	8.40
10	2 x 10	22.04	30.30	26.59	25.74	26.28	9.44	10.04	6.79	5.95	8.21
11	3 x 6	23.88	32.53	28.51	-	28.28	5.82	4.65	2.36	1.77	3.23
12	3 x 7	21.48	27.99	29.91	28.67	27.24	6.48	6.83	4.07	2.63	4.78
13	3 x 8	24.30	27.95	28.75	28.36	27.76	7.73	6.63	4.49	3.53	5.38
14	3 x 9	23.58	27.69	29.60	24.69	26.42	9.21	9.29	7.25	6.03	8.04
15	3 x 10	22.46	29.25	21.46	25.93	24.78	10.4	9.22	6.74	5.01	7.95
16	4 x 6	20.50	29.49	-	25.39	25.49	6.51	6.10	2.18	1.95	3.80
17	4 x 7	22.87	27.30	25.38	24.57	25.06	6.42	5.38	3.22	2.77	4.15
18	4 x 8	23.51	28.62	26.18	22.22	25.02	6.51	6.63	4.32	4.4	5.37
19	4 x 9	23.47	23.19	26.88	25.67	24.96	8.76	8.76	4.50	6.45	7.20
20	4 x 10	24.94	27.93	30.24	25.21	27.22	9.35	8.99	6.41	3.76	7.16

Table A.1 continued

Hybrid	Cross	Zn (µg/g)					GY (t ha-1)				
		Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average	Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average
21	5 x 6	27.83	31.50	26.43	-	28.66	3.84	4.72	1.79	1.37	2.48
22	5 x 7	23.47	23.74	25.79	26.59	25.08	4.47	4.63	3.29	2.70	3.40
23	5 x 8	26.06	32.25	25.42	29.16	28.62	5.95	5.60	4.60	2.91	4.62
24	5 x 9	23.02	24.16	24.85	26.86	24.75	10.72	9.43	6.23	6.12	8.29
25	5 x 10	23.28	29.81	25.22	24.99	25.94	10.24	10.98	6.32	5.30	8.30
26	1 x 11	23.09	27.63	26.55	21.56	24.56	10.04	9.78	7.57	4.16	7.91
27	1 x 12	21.74	23.83	20.91	22.73	22.11	9.88	10.37	7.71	6.14	8.68
28	1 x 13	24.26	29.36	26.80	27.08	27.11	9.19	9.17	7.34	4.80	7.63
29	1 x 14	23.58	23.80	24.72	23.54	23.95	9.25	9.40	6.42	5.67	7.85
30	1 x 15	21.25	24.18	21.94	21.60	22.01	9.10	8.90	7.15	3.92	7.25
31	2 x 11	23.81	27.84	27.17	22.96	25.45	9.78	9.64	7.97	5.13	8.24
32	2 x 12	23.77	29.29	27.09	21.21	25.36	9.91	10.06	7.84	6.39	8.64
33	2 x 13	25.09	30.89	28.00	24.44	27.27	9.97	10.32	8.87	6.27	9.05
34	2 x 14	24.90	25.30	23.83	24.25	24.61	9.08	8.42	7.51	6.39	7.89
35	2 x 15	21.03	26.61	25.14	26.39	24.78	10.10	8.79	8.36	5.36	8.23
36	3 x 11	22.98	25.15	23.02	21.49	22.98	9.25	11.02	6.42	4.83	7.88
37	3 x 12	23.17	26.81	20.99	21.94	23.13	10.48	9.57	7.19	5.79	8.28
38	3 x 13	22.76	25.80	25.70	20.64	23.54	9.16	10.77	7.71	5.98	8.52
39	3 x 14	20.99	22.68	20.99	23.59	21.69	8.45	7.84	6.92	4.39	6.80
40	3 x 15	20.43	21.86	23.85	21.88	21.59	9.92	9.32	8.16	4.52	7.96
41	4 x 11	23.43	28.66	25.78	21.50	24.88	9.12	9.05	7.44	4.57	7.45
42	4 x 12	22.46	25.59	22.53	22.92	23.20	9.21	9.24	7.64	5.72	7.97
43	4 x 13	24.60	30.16	25.82	26.74	26.95	8.87	10.73	8.55	6.89	8.90
44	4 x 14	19.03	26.05	22.57	22.65	22.31	11.15	9.19	6.64	7.06	8.64
45	4 x 15	21.29	24.27	22.65	22.08	22.28	9.09	9.48	7.57	5.60	7.96

Table A.1 continued.

Hybrid	Cross	Zn (µg/g)					GY (t ha-1)				
		Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average	Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average
46	5 x 11	21.85	29.51	23.27	20.73	23.70	8.79	8.93	6.97	5.93	7.71
47	5 x 12	24.26	26.61	21.57	23.06	23.80	7.64	9.46	7.88	5.07	7.53
48	5 x 13	27.64	25.07	27.26	NA	26.62	9.13	9.81	7.02	4.44	7.69
49	5 x 14	20.50	25.75	22.86	20.86	22.18	6.26	8.39	6.74	7.08	7.13
50	5 x 15	20.50	23.58	23.52	22.59	22.34	9.96	9.90	7.43	5.21	8.15
51	1 x 16	26.78	31.35	33.33	30.46	31.07	9.73	11.07	7.74	5.80	8.80
52	1 x 17	23.66	26.82	27.65	24.81	25.86	9.44	10.53	6.36	5.25	8.05
53	1 x 18	23.70	28.98	30.70	26.51	27.62	11.79	9.94	7.71	4.68	8.58
54	1 x 19	22.61	25.78	28.24	24.77	25.43	9.98	10.00	7.69	4.98	8.26
55	1 x 20	25.39	29.69	32.80	25.61	29.07	10.01	11.24	7.70	4.90	8.61
56	2 x 16	28.32	28.96	32.94	32.58	31.45	10.35	9.14	6.04	6.79	8.22
57	2 x 17	25.27	30.20	31.90	28.21	29.26	9.80	10.12	5.92	4.71	7.80
58	2 x 18	25.46	30.19	23.38	27.20	26.84	10.84	9.85	7.34	4.94	8.35
59	2 x 19	26.25	29.98	25.67	25.50	27.03	10.20	11.03	7.69	5.37	8.79
60	2 x 20	25.80	32.76	35.38	25.79	30.25	11.18	9.51	8.93	7.31	9.40
61	3 x 16	22.61	30.51	32.32	24.13	27.41	9.30	9.97	7.12	6.86	8.41
62	3 x 17	22.08	27.97	28.96	25.54	26.18	9.23	10.03	6.52	4.00	7.43
63	3 x 18	20.95	30.77	23.71	24.88	25.08	11.64	10.68	7.91	5.19	8.93
64	3 x 19	22.72	29.77	28.90	27.31	27.37	9.17	10.61	6.91	4.77	7.88
65	3 x 20	24.07	27.60	31.95	27.87	28.16	10.20	10.93	6.70	4.80	8.29
66	4 x 16	25.54	27.84	31.99	27.39	28.61	10.40	8.74	6.81	6.65	8.15
67	4 x 17	24.56	30.72	23.97	24.47	26.05	8.76	7.70	6.18	5.48	6.99
68	4 x 18	21.93	23.45	28.67	27.16	25.30	11.74	9.95	8.02	4.85	8.81
69	4 x 19	26.33	30.56	26.87	26.01	27.78	10.00	10.98	7.77	4.74	8.49
70	4 x 20	22.76	27.88	29.35	24.71	26.35	12.59	10.39	8.24	5.36	9.31
71	5 x 16	25.58	29.74	25.01	28.29	27.44	8.67	8.81	7.10	6.47	7.81
72	5 x 17	26.14	27.68	26.72	24.84	26.47	9.30	9.52	6.29	5.48	7.74

Table A.1 continued

Hybrid	Cross	Zn (µg/g)					GY (t ha-1)				
		Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average	Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average
73	5x 18	23.55	28.46	26.26	27.35	26.57	11.03	9.16	8.40	5.78	8.63
74	5 x 19	24.49	30.25	29.37	21.66	26.72	11.74	9.39	7.62	5.52	8.64
75	5 x 20	25.35	29.26	29.39	25.73	27.64	11.15	10.37	7.00	5.61	8.71
76	6 x 11	21.10	26.29	24.39	21.99	23.27	7.54	7.50	5.66	3.63	5.99
77	6 x 12	20.05	23.67	21.61	23.93	21.99	10.41	11.08	6.47	4.32	8.21
78	6 x 13	21.14	25.45	21.59	24.28	22.81	9.37	9.89	6.81	4.16	7.55
79	6 x 14	19.00	23.57	21.24	20.43	20.73	7.28	9.47	5.92	5.04	6.94
80	6 x 15	18.92	24.34	22.53	21.24	21.35	10.05	8.64	5.98	5.69	7.61
81	7 x 11	22.79	25.25	22.40	21.29	22.71	8.76	7.93	5.72	3.73	6.53
82	7 x 12	21.55	22.65	21.61	19.71	21.05	8.28	8.60	6.69	5.43	7.33
83	7 x 13	20.76	25.02	22.91	24.12	23.03	7.71	8.58	6.63	5.20	7.02
84	7 x 14	18.85	20.49	20.53	20.01	19.61	7.59	6.52	5.96	4.54	6.01
85	7 x 15	18.47	20.79	19.04	19.45	18.93	7.40	7.22	6.85	3.83	6.27
86	8 x 11	21.24	23.32	24.32	21.80	22.41	10.81	9.77	7.83	5.89	8.66
87	8 x 12	20.84	24.18	22.77	23.27	22.51	9.64	9.14	7.30	4.18	7.66
88	8 x 13	23.58	27.66	25.14	24.69	25.32	9.03	10.08	7.45	6.03	8.32
89	8 x 14	21.67	23.14	23.19	23.97	22.70	9.94	7.79	6.88	6.32	7.77
90	8 x 15	20.09	22.70	20.31	22.29	20.88	9.29	7.93	7.71	6.07	7.74
91	9 x 11	18.88	21.91	24.43	22.67	21.58	5.55	6.69	4.47	4.30	5.08
92	9 x 12	18.92	25.10	21.61	23.41	21.94	8.53	8.39	4.21	4.74	6.44
93	9 x 13	22.12	28.68	26.22	26.78	26.00	9.92	9.29	7.58	5.95	8.28
94	9 x 14	18.55	22.04	20.71	22.14	20.56	5.14	6.06	3.80	3.28	4.47
95	9 x 15	18.06	20.43	20.04	21.32	19.54	7.37	7.55	4.36	5.20	6.12
96	10 x 11	21.48	25.06	25.51	21.65	23.27	7.46	8.59	5.60	4.93	6.61
97	10 x 12	17.98	24.33	22.15	24.10	21.67	5.27	6.49	3.01	1.91	3.84
98	10 x 13	21.44	30.12	24.14	22.95	24.60	8.73	9.20	5.29	5.50	7.33
99	10 x 14	20.69	21.73	21.53	22.02	21.17	6.19	10.57	7.02	7.19	7.83

Table A.1 continued.

Hybrid	Cross	Zn (µg/g)					GY (t ha-1)				
		Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average	Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average
100	10 x 15	21.97	23.06	19.58	22.69	21.43	6.08	8.27	5.52	4.94	6.15
101	6 x 16	23.77	26.30	29.33	26.13	26.46	9.72	8.27	6.30	4.88	7.36
102	6 x 17	20.73	25.52	23.98	24.48	23.51	8.95	9.05	6.65	4.37	7.31
103	6 x 18	22.61	30.35	24.35	26.53	26.15	10.65	10.92	8.16	4.57	8.68
104	6 x 19	23.77	26.60	24.15	24.54	24.82	11.39	10.40	6.38	5.36	8.54
105	6 x 20	24.15	28.10	26.24	26.83	26.50	10.09	9.31	8.55	5.68	8.55
106	7 x 16	24.60	27.32	26.22	27.38	26.70	8.63	8.74	6.52	5.74	7.51
107	7 x 17	20.24	24.41	24.35	23.76	23.01	7.18	7.11	4.98	3.60	5.64
108	7 x 18	20.46	27.51	26.88	25.92	25.15	9.16	8.74	6.72	6.59	7.87
109	7 x 19	20.88	26.19	25.14	26.37	24.64	8.12	8.57	5.76	5.32	7.02
110	7 x 20	22.76	27.08	30.17	23.03	25.92	9.50	9.87	6.89	5.20	7.91
111	8 x 16	25.43	29.75	28.21	22.01	26.47	10.40	10.32	7.55	6.18	8.81
112	8 x 17	23.88	25.83	24.60	27.40	25.73	9.49	10.21	5.97	5.02	7.65
113	8 x 18	22.31	23.81	28.17	28.51	25.76	12.05	10.29	5.60	4.48	8.23
114	8 x 19	24.71	28.22	25.35	22.58	25.32	11.39	9.14	6.53	5.78	8.28
115	8 x 20	23.47	27.29	28.96	NA	26.33	11.34	10.20	8.06	3.43	8.26
116	9 x 16	23.21	23.79	26.70	25.07	24.72	6.81	5.34	2.24	5.17	4.83
117	9 x 17	22.98	26.43	25.57	24.21	24.80	5.15	5.65	2.02	2.95	3.66
118	9 x 18	19.00	25.33	27.79	24.27	23.98	7.67	9.38	6.55	5.45	7.25
119	9 x 19	23.40	26.89	25.51	24.47	25.07	9.47	8.58	6.42	5.70	7.60
120	9 x 20	22.49	24.68	27.06	NA	24.51	8.84	9.76	6.70	6.78	8.18
121	10 x 16	23.24	29.25	25.01	23.06	25.14	9.58	8.11	3.83	4.19	6.48
122	10 x 17	19.11	30.05	26.07	22.56	24.27	8.19	8.54	4.38	4.79	6.42
123	10 x 18	24.57	32.77	26.80	22.92	27.01	4.79	3.85	1.39	3.48	3.20
124	10 x 19	20.50	24.15	27.71	29.79	25.49	6.34	4.23	1.08	1.53	2.92

Table A.1 continued.

Hybrid	Cross	Zn (µg/g)					GY (t ha-1)				
		Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average	Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Average
125	10 x 20	24.41	28.76	31.74	25.27	27.79	4.33	3.56	1.20	1.90	2.33
126	11 x 16	24.18	28.56	27.42	25.32	26.73	9.10	6.72	5.80	4.51	6.50
127	11 x 17	20.91	26.58	28.17	23.12	24.59	7.16	8.59	6.27	4.54	6.55
128	11 x 18	18.47	25.29	22.90	21.90	21.79	9.53	9.78	6.46	4.07	7.46
129	11 x 19	20.73	25.36	23.85	22.87	22.99	8.87	8.18	7.00	5.89	7.52
130	11 x 20	21.82	26.08	24.93	23.52	23.98	9.38	8.77	6.70	5.35	7.58
131	12 x 16	22.15	24.97	24.81	25.11	24.29	8.05	9.36	5.81	4.16	6.82
132	12 x 17	20.65	23.03	22.88	25.19	22.73	5.41	7.48	4.80	3.95	5.17
133	12 x 18	19.67	22.42	20.41	22.41	20.91	8.97	6.47	3.71	3.57	5.54
134	12 x 19	18.88	21.64	20.87	22.39	20.43	7.37	7.89	3.94	3.12	5.40
135	12 x 20	20.39	25.01	24.56	23.36	23.15	7.67	7.40	4.67	4.27	6.03
136	13 x 17	24.15	25.89	25.26	26.47	25.57	7.59	9.75	6.82	5.78	7.56
137	13 x 18	20.99	25.39	27.55	24.72	24.54	10.57	9.53	8.01	6.13	8.65
138	13 x 19	22.34	27.56	24.48	22.88	24.25	9.06	7.81	7.03	4.85	7.08
139	13 x 20	22.49	27.80	27.75	25.67	25.98	10.14	9.77	6.49	6.72	8.48
140	14 x 16	20.84	22.37	28.13	25.86	24.02	7.47	6.62	4.46	3.02	5.16
141	14 x 17	20.43	21.76	23.02	24.33	22.19	6.76	7.60	4.22	3.48	5.36
142	14 x 18	19.19	22.38	20.70	20.74	20.37	8.64	8.89	5.66	4.22	6.79
143	14 x 19	20.31	21.71	22.07	21.24	20.99	8.38	7.49	4.35	5.07	6.29
144	14 x 20	18.92	22.68	24.68	23.94	22.08	9.05	8.54	5.81	4.62	7.07
145	15 x 16	18.81	23.58	20.74	20.98	20.76	8.43	7.28	7.21	6.19	7.33
146	15 x 18	18.13	22.35	22.75	21.63	20.87	9.24	8.64	7.64	7.16	8.28
147	15 x 19	22.23	22.86	21.40	20.96	21.60	8.26	7.79	6.66	5.03	7.01
148	15 x 20	19.56	23.46	22.57	23.79	22.00	8.98	8.49	5.31	5.05	7.04
149	ARTILLERO	17.83	22.17	21.74	21.43	20.34	9.25	6.31	6.15	6.50	7.09
150	SULTAN	19.07	20.52	25.67	19.94	20.59	9.61	6.31	5.17	5.21	6.55

Table A.2 Phenotypic correlation between kernel Zn concentration and all agronomic traits

Trait	Tlaltizapan 2015	Tlaltizapan 2016	Agua Fria 2015	Cotaxtla 2015	Across four environments
Grain yield	0.12	0.09	0.03	-0.09	0.02
Plant height	-0.04	-0.09	0.00	-0.14*	-0.01
Days to anthesis	0.20***	0.17**	0.17*	0.16*	-0.06
Anthesis silking interval	-0.03	0.05	0.05	-	0.03
Days to silking	0.19**	0.16**	0.17**	0.16*	-0.05

*, ** and ***significant at $p < 0.05$,0.01 and 0.001 probability levels, respectively.

Table A.3 Analysis of variance of combining abilities and their interactions for all traits

Source of variation	df	Set1	Set2	Set3	Set4	Set5	Set6
		Group A x Group B	Group A x Group C	Group A x Group D	Group B x Group C	Group B x Group D	Group C x Group D
Grain yield							
GCA_f	4	2.63	3.13	0.09	21.96**	85.95***	24.3***
GCA_m	4	128.34***	2.16	8.96	7.07*	4.13	2.97
SCA	16	6.47**	1.86	1.23	8.59***	15.1***	2.32*
Env	3	129.96**	135.53*	214.92**	120.65*	172.31*	105.88*
Env*GCA_m	12	1.42	3.85***	3.49***	1.84	1.70	3.27*
Env*GCA_f	12	1.44	2.29*	1.78	2.78	5.61**	0.94
Env*SCA	48	1.16	1.09	1.01	1.64	1.92*	1.22
Rep (Env)	3	5.00**	9.25**	6.50***	13.0***	11.52***	10.84***
Blk (Rep)	28	0.74	1.45	1.37	1.05	1.46	1.17
Residual Error	68	1.19	1.90	1.01	1.40	1.21	1.27
Zn concentration							
GCA_f	4	13.81	57.10***	28.65*	9.69	17.54	71.03***
GCA_m	4	22.86*	79.97**	57.23*	106.5***	27.49	34.26***
SCA	16	11.25	5.13	14.84	7.90*	7.39	4.88
Env	3	93.18	81.77*	149.57	65.17	146.33*	75.44*
Env*GCA_m	12	6.43	10.14	13.63	9.32*	9.2	2.89
Env*GCA_f	12	12.43	3.61	8.82	6.18	12.95	5.41
Env*SCA	45	13.81	7.45**	14.3**	3.85	11.38*	4.92*
Rep (Env)	3	12.47	2.65	4.65	10.41	6.56	6.85
Blk (Rep)	28	5.17	4.44	7.49	4.71	4.86	3.34
Residual Error	53	10.02	3.86	6.59	4.22	6.99	3.05

Table A.3 continued

Source of variation	df	Set1 Group A x Group B	Set2 Group A x Group C	Set3 Group A x Group D	Set4 Group B x Group C	Set5 Group B x Group D	Set6 Group C x Group D
Days to anthesis							
GCAf	4	4.89*	4.06*	2.21	6.67*	3.52	28.17**
GCAm	4	13.24**	16.46*	3.88	19.88***	6.65**	1.82*
SCA	16	1.38	0.65	0.92	3.47***	4.14**	2.52
Env	3	1193.15***	1140.47***	1402.17***	1185.24***	1363.55**	1075.8***
Env*GCA_m	12	2.34	2.78*	2.77***	0.79	0.78	0.36
Env*GCA_f	12	1.2	0.81	1.53*	1.38	2.22	3.59**
Env*SCA	48	1.39	1.45	0.80	1.12	1.51	1.40
Rep (Env)	3	0.88	3.42*	6.12***	3.65*	3.65	3.30*
Blk (Rep)	28	0.85	1.05	0.92	0.81	1.36	0.72
Residual Error	68	0.97	1.22	0.92	1.22	1.59	1.04
Days to silking							
GCA_f	4	7.13*	5.38*	2.27	5.32**	4.51	36.95**
GCA_m	4	15.82**	26.55*	9.32	32.02***	11.4**	5.11*
SCA	16	1.57	0.67	1.34	5.32***	6.17**	2.39
Env	3	1179.56***	1149.31***	1389.7***	1161.64***	1391.77***	1085.06***
Env*GCA_m	12	2.22	5.62***	4.03**	2.32	2.02	1.32
Env*GCA_f	12	1.99	1.03	1.81	0.82	5.06*	4.69**
Env*SCA	48	1.74	1.54	1.44	1.48	2.20	1.38
Rep (Env)	3	0.68	2.36	5.07**	3.61*	2.02	2.72
Blk (Rep)	28	1.06	1.27	1.22	0.98	1.79	1.26
Residual Error	68	1.7	1.56	1.07	1.22	1.82	1.18

*, ** and *** significant at $p < 0.05$, 0.01 and 0.001, respectively. GC_{Af} = the general combining ability effect of the lines designated as females; GCAm = the general combining ability effect of the lines designated as males. Group A, B, C and D = high-Zn QPM, low-Zn QPM, low-Zn non-QPM and high-Zn non-QPM, respectively.

Table A.4 General combining ability (GCA) effects for kernel Zn concentration at each environment

Inbred line	Tlaltizapan 2015			Tlaltizapan 2016			Agua Fria 2015			Cotaxtla 2015		
High Zn, QPM	LZn, QPM	LZn, non-QPM	HZn, non-QPM	LZn, QPM	LZn, non-QPM	HZn, non-QPM	LZn, QPM	LZn, non-QPM	HZn, non-QPM	LZn, QPM	LZn, non-QPM	HZn, non-QPM
1	1.28	0.00	-0.06	0.00	-0.45	-0.06	0.00	0.01	0.09	-0.20	0.14	0.00
2	-0.49	0.09	1.97*	0.00	1.47	0.13	0.00	1.88*	0.05	0.24	0.37	0.02
3	-0.65	-0.06	-2.24*	0.00	-1.52	0.03	0.00	-1.18	0.01	0.37	-0.42	-0.01
4	-0.71	-0.05	-0.29	0.00	0.61	-0.10	0.00	-0.27	-0.05	-0.79	0.17	0.00
5	0.57	0.02	0.61	0.00	-0.11	0.00	0.00	-0.44	-0.10	0.38	-0.27	-0.01
Low Zn, QPM	HZn, QPM	LZn, non-QPM	HZn, non-QPM	HZn, QPM	LZn, non-QPM	HZn, non-QPM	HZn, QPM	LZn, non-QPM	HZn, non-QPM	HZn, QPM	LZn, non-QPM	HZn, non-QPM
6	0.00	-0.24	0.19	1.73	0.23	0.19	0.00	-0.03	0.00	0.00	-0.13	0.00
7	0.00	0.06	-0.44	-1.57	-0.42	-0.34	0.00	-0.28	0.00	0.00	-0.86	0.00
8	0.00	0.70	0.68	0.46	0.06	-0.07	0.00	0.20	0.00	0.00	0.43	0.00
9	0.00	-0.72	-0.22	-1.91	-0.16	-1.00	0.00	0.06	0.00	0.00	0.48	0.00
10	0.00	0.20	-0.21	1.28	0.29	1.22	0.00	0.05	0.00	0.00	0.07	0.00
Low Zn, non-QPM	HZn, QPM	LZn, QPM	HZn, non-QPM	HZn, QPM	LZn, QPM	HZn, non-QPM	HZn, QPM	LZn, QPM	HZn, non-QPM	HZn, QPM	LZn, QPM	HZn, non-QPM
11	0.32	0.61	0.44	1.61	0.41	2.35	1.04	1.94	1.44	-0.78	-0.31	-0.62
12	0.37	-48.00	-0.42	0.21	-0.06	-1.08	-1.64	-0.45	-1.31	-0.28	0.07	0.17
13	2.31*	1.26	1.74*	2.18	3.69**	2.73*	2.67*	1.72	2.38*	1.06	1.10	2.38*
14	-1.01	-0.58	-0.83	-1.67	-2.11	-2.47*	-1.27	-1.01	-0.50	0.10	-0.09	-0.73
15	-1.99	-0.81	-0.94	-2.33	-1.94	-1.52	-0.81	-2.21*	-2.02	-0.09	-0.28	-1.19

Table A.4 continued.

Inbred line	Tlaltizapan 2015			Tlaltizapan 2016			Agua Fria 2015			Cotaxtla 2015		
High Zn, non-QPM	HZn, QPM	LZn, QPM	LZn, non-QPM	HZn, QPM	LZn, QPM	LZn, non-QPM	HZn, QPM	LZn, QPM	LZn, non-QPM	HZn, QPM	LZn, QPM	LZn, non-QPM
16	1.18	1.22	0.81	0.00	-0.66	0.52	1.80	0.41	1.04	1.57	0.00	1.10
17	-0.12	-1.12	0.45	0.00	-1.36	-0.17	-1.01	-1.66	0.30	-0.64	0.00	1.11
18	-1.24	-0.86	-1.24	0.00	2.45	-0.42	-2.12	0.19	-0.87	0.18	0.00	-1.28
19	0.00	0.01	0.10	0.00	-0.55	-0.28	-1.01	-1.01	-1.14	-0.90	0.00	-1.46
20	0.18	0.71	-0.12	0.00	0.72	0.37	2.35	2.06	0.67	-0.21	0.00	0.53

*, ** and *** Significant at p <0.05, 0.01 and 0.001, respectively.

Lines from one group were mated in a modified design scheme to lines from three other groups so that three independent estimates of combining ability were computed for each line. LZn, QPM and LZn, non-QPM= low zinc QPM and non-QPM lines, respectively; HZn, QPM and HZn, non-QPM =high Zn QPM and non QPM lines, respectively.

Table A.5 Specific combining ability for Zn among sets of hybrids evaluated in four environments

Set1 (Group A x B)			Set2 (Group A x C)			Set3 (Group A x D)			Set4 (Group B x C)			Set5 (Group B x D)			Set6 (Group C x D)		
F	M	SCA	F	M	SCA	F	M	SCA	F	M	SCA	F	M	SCA	F	M	SCA
1	6	0.20	1	11	0.00	1	16	0.34	6	11	0.27	6	16	0.00	11	16	0.69
1	7	-0.04	1	12	0.00	1	17	-0.21	6	12	0.04	6	17	0.00	11	17	0.14
1	8	0.06	1	13	0.00	1	18	0.15	6	13	-0.57	6	18	0.00	11	18	-0.46
1	9	-0.06	1	14	0.00	1	19	-0.30	6	14	-0.11	6	19	0.00	11	19	-0.07
1	10	0.15	1	15	0.00	1	20	0.11	6	15	0.37	6	20	0.00	11	20	-0.16
2	6	-0.09	2	11	0.00	2	16	0.27	7	11	0.29	7	16	0.00	12	16	0.32
2	7	0.17	2	12	0.00	2	17	0.23	7	12	-0.07	7	17	0.00	12	17	-0.01
2	8	-0.01	2	13	0.00	2	18	-0.12	7	13	-0.24	7	18	0.00	12	18	-0.14
2	9	0.05	2	14	0.00	2	19	-0.16	7	14	-0.35	7	19	0.00	12	19	-0.47
2	10	-0.03	2	15	0.00	2	20	0.19	7	15	-0.43	7	20	0.00	12	20	0.21
3	6	0.16	3	11	0.00	3	16	-0.18	8	11	-0.25	8	16	0.00	13	17	0.01
3	7	0.11	3	12	0.00	3	17	-0.05	8	12	0.10	8	17	0.00	13	18	0.20
3	8	0.12	3	13	0.00	3	18	-0.17	8	13	0.30	8	18	0.00	13	19	-0.07
3	9	0.03	3	14	0.00	3	19	0.14	8	14	0.48	8	19	0.00	13	20	0.18
3	10	-0.25	3	15	0.00	3	20	0.08	8	15	-0.05	8	20	0.00	14	16	0.30
4	6	-0.14	4	11	0.00	4	16	0.03	9	11	-0.42	9	16	0.00	14	17	-0.03
4	7	-0.16	4	12	0.00	4	17	-0.07	9	12	0.08	9	17	0.00	14	18	-0.21
4	8	-0.24	4	13	0.00	4	18	-0.12	9	13	0.75	9	18	0.00	14	19	-0.08
4	9	-0.15	4	14	0.00	4	19	0.20	9	14	-0.15	9	19	0.00	14	20	-0.12
4	10	0.13	4	15	0.00	4	20	-0.22	9	15	-0.38	9	20	0.00	15	16	-0.89
5	6	0.21	5	11	0.00	5	16	-0.18	10	11	0.19	10	16	0.00	15	18	0.23
5	7	-0.19	5	12	0.00	5	17	-0.01	10	12	-0.19	10	17	0.00	15	19	0.42
5	8	0.25	5	13	0.00	5	18	0.07	10	13	0.08	10	18	0.00	15	20	0.03
5	9	-0.20	5	14	0.00	5	19	0.01	10	14	-0.02	10	19	0.00	-	-	-
5	10	-0.08	5	15	0.00	5	20	-0.02	10	15	0.26	10	20	0.00	-	-	-

Set1 = High-Zn QPM crossed to low-Zn QPM, Set2 = High-Zn QPM crossed to low-Zn non-QPM, Set3 =High-Zn QPM crossed to high-Zn non-QPM, Set4 =Low-Zn QPM crossed to low-Zn non-QPM, Set5 = High-Zn QPM crossed to high-Zn non-QPM and Set6 = Low-Zn non-QPM crossed to high-Zn non-QPM. F, M and SCA= Female, Male and Specific combining ability, respectively.

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